DEVELOPMENT OF INTRA- AND INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER PROTOCOLS USING EAR FIBROBLAST CELLS AS DONOR KARYOPLASTS FOR PRODUCTION OF CLONED CAPRINE EMBRYOS

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FACULTY OF SCIENCE
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KUALA LUMPUR

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Field of Study	:	Reproductive Biotechnology

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ABSTRACT

In Malaysia, the application of various assisted reproductive technologies (ART), particularly reproductive cloning, in goat production system, is still at the infancy stage. Thus, this pioneering study was conducted with the aim to produce cloned caprine embryos through intraspecies somatic cell nuclear transfer (intraspSCNT) and interspecies somatic cell nuclear transfer (interspSCNT) techniques using caprine ear skin fibroblast cell as donor karyoplast as well as developing a protocol for caprine SCNT (intraspSCNT and interspSCNT). The caprine intraspSCNT and interspSCNT (caprine karyoplast-bovine cytoplast) studies were carried out using caprine and bovine oocytes obtained from the abattoir-derived ovaries or via laparoscopic ovum pick-up (LOPU) technique on superstimulated does. The collected oocytes were subsequently cultured in *in vi*tro maturation (IVM) medium according to the optimised duration. The matured oocytes were then subjected to enucleation process. This was followed by the transfer of caprine ear skin fibroblast cell into the enucleated oocytes. The couplets were electrofused and chemically activated before *in vitro* cultured in CO_2 (5%) incubator at 38.5°C in humidified atmosphere for 8 days. The cloned blastocyst was stained with Hoechst 33342 dye for blastomere enumeration. The data were presented as mean±SEM and were analysed using one-way ANOVA. The significant differences among treatments were further analysed by DMRT and $P<0.05$ was considered significant.

In Experiment 1, the effect of different sources of gonadotrophin (PMSG versus pFSH) on caprine superstimulatory responses was evaluated. Both PMSG and pFSH employed in the designated regimes have relatively similar potential to stimulate caprine ovaries for oocyte retrieval via LOPU with the average oocyte yield of 11 and 12 oocytes per doe, respectively. However, the efficacy of PMSG could not surpass the pFSH, particularly when it was employed in the repeated ovarian stimulation and oocyte

retrieval protocols. This was indicated by the lower number of oocyte yielded from PMSG treated group than pFSH ($P<0.05$) group at the third OR cycle.

In Experiment 2, the effect of different sources of caprine oocytes (LOPU- versus abattoir-derived ovaries) on the oocyte yield, grades and maturation performance was investigated. Oocyte retrieval from LOPU source produced better quality oocytes (Grades A and B) (39 to 40%) compared to abattoir source (18 to 32%), even though the oocyte yield was lower in LOPU. Correspondingly, caprine oocytes from LOPU gave higher maturation rate than abattoir-derived ovaries (79.6% versus 69.7%, respectively) when the optimised IVM duration was used. The optimum IVM durations for caprine oocytes retrieved from LOPU- and abattoir derived-ovaries determined in this laboratory setting were 18 to 22 hours and 22 to 26 hours, respectively.

In Experiment 3, production of cloned bovine and gaur embryos via intraspSCNT and interspSCNT approaches was carried out as a preliminary study for caprine SCNT research. Both cloned bovine and gaur embryos could be produced *in vitro* up to hatched blastocyst stage with no significant difference ($P>0.05$) in their developmental rate (18.6% and 19%, respectively).

In Experiment 4, improvement on the *in vitro* cloned caprine embryos production by considering the effects of maturation duration, activation treatment and *in vitro* culture protocol was carried out. Caprine oocytes from superstimulated does which was matured at 18 to 22 hours gave a significantly ($P<0.05$) higher maturation rate, enucleation rate and IVD rates (2-cell to morula stage) than oocytes which was matured at 23 to 27 hours after intraspSCNT. Both activation protocols [(7% EtOH + CD-CHX) and (CaI + 6-DMAP)] had comparable efficiency in inducing the development of caprine reconstructed embryos. KSOMaa basal medium supported the *in vitro* development of cloned caprine embryos better than mSOFaa in the one-step culture system as cloned blastocyst could only be developed when cultured in KSOMaa as in

this study. Increasing glucose supplementation to 2.78 mM in KSOMaa medium at Day 2 of *in vitro* culture (IVC) enhanced the cloned caprine blastocyst rate (19.9%) and promoted hatching of blastocyst (15.6%).

In Experiment 5, the efficacy of producing cloned caprine embryos using intraspecies SCNT (intraspSCNT) versus interspecies SCNT (interspSCNT) approaches was evaluated. Both intraspSCNT and interspSCNT approaches enabled production of cloned caprine blastocyst with the rate of 17.3% and 8.3%, respectively. However, the efficiency of interspSCNT approach was still low compared to the intraspSCNT approach. No pregnancy was detected after the attempt of embryo transfer on the cloned caprine embryos.

In a nutshell, the present study resulted in several novel findings in Malaysia animal reproduction research scenario. This includes the success in producing cloned caprine embryos up to hatched blastocyst stage through intraspSCNT and interspSCNT (caprine karyoplast-bovine cytoplast) approaches using caprine ear skin fibroblast cell; the discovery of a new IVC system that could support the caprine embryos *in vitro* development to blastocyst stage (using KSOMaa at Day 0 to Day 2 and increasing the glucose supplementation to 2.78 mM in KSOMaa for Day 2 to Day 8 of *in vitro* culture); protocols for caprine intraspSCNT and interspSCNT were developed and could be served as references for future studies related to goat somatic cell nuclear transfer in this laboratory.

ABSTRAK

Di Malaysia, aplikasi pelbagai teknologi reproduksi berbantu (ART), terutamanya teknologi pengklonan, dalam sistem produksi kambing, masih pada tahap permulaan. Maka, kajian julung kali ini telah dilakukan dengan matlamat untuk menghasilkan klon embrio kaprin melalui teknik pemindahan nukleus sel somatik intraspesies-(intraspSCNT) dan interspesies (interspSCNT) dengan menggunakan sel fibroblas kulit telinga kaprin sebagai penderma karioplas dan juga membangunkan satu protokol untuk SCNT kaprin (intraspSCNT dan interspSCNT). Kajian intraspSCNT dan interspSCNT (karioplas kaprin-sitoplas bovin) kaprin telah dijalankan dengan menggunakan oosit kaprin dan bovin yang diperolehi daripada ovari diambil dari rumah sembelihan atau melalui teknik pungutan ovum secara laparoskopik (LOPU) ke atas kambing betina yang disuperstimulasikan. Oosit yang dikumpulkan kemudiannya dikultur dalam medium pematangan *in vitro* (IVM) mengikut tempoh masa yang telah dioptimumkan. Oosit matang kemudiannya telah melalui proses enukleasi. Ini diikuti dengan pemindahan sel fibroblast kulit telinga kaprin ke dalam oosit yang telah dienukleasikan. Kuplet dielektrofusikan dan diaktifkan secara kimia sebelum dikultur *in vitro* dalam inkubator CO_2 (5%) pada suhu 38.5°C dalam suasana atmosfera lembap selama 8 hari. Blastosis klon diwarnakan dengan pewarna Hoechst 33342 untuk penghitungan blastomer. Data telah dibentangkan sebagai min±SEM dan dianalisis dengan menggunakan ANOVA sehala. Perbezaan yang signifikan di antara perlakuan telah dianalisis selanjutnya dengan DMRT dan $P<0.05$ dianggap sebagai signifikan.

Dalam Eksperimen 1, kesan sumber gonadotropin yang berbeza (PMSG lawan pFSH) terhadap respons superstimulasi kaprin telah dinilai. Kedua-dua PMSG dan pFSH yang digunakan dalam rejim yang direkabentuk mempunyai potensi yang agak sama untuk stimulasi ovari kaprin bagi perolehan oosit melalui LOPU dengan purata

perolehan oosit masing-masing sebanyak 11 dan 12 oosit per kambing betina. Walau bagaimanapun, keberkesanan PMSG tidak dapat memintasi pFSH, terutamanya bila ia digunakan dalam protokol stimulasi ovari dan perolehan oosit yang berulang. Ini telah ditunjukkan melalui bilangan oosit yang diperolehi yang lebih rendah daripada kumpulan disuntik PMSG berbanding kumpulan pFSH ($P<0.05$) di kitar OR ketiga.

Dalam Eksperimen 2, kesan sumber oosit kaprin yang berbeza (LOPU- lawan ovari daripada rumah sembelihan) terhadap perolehan oosit, gred dan prestasi kematangan telah dikaji. Oosit yang diperolehi daripada sumber LOPU menghasilkan kualiti oosit yang lebih baik (Gred A dan B) (39 ke 40%) berbanding dengan sumber rumah sembelihan (18 ke 32%), walaupun pemerolehan oosit adalah rendah bagi LOPU. Sejajar dengan itu, oosit kaprin daripada LOPU memberi kadar pematangan yang lebih tinggi daripada ovari yang diperolehi daripada rumah sembelihan (masing-masing 79.6% lawan 69.7%) apabila tempoh IVM yang dioptimumkan telah digunakan. Tempoh IVM yang optimum bagi oosit kaprin yang diperolehi daripada LOPU- dan ovari daripada rumah sembelihan yang ditentukan dalam suasana makmal ini adalah masing-masing 18 hingga 22 jam dan 22 hingga 26 jam.

Dalam Eksperimen 3, penghasilan embrio bovin dan gaur klon melalui pendekatan intraspSCNT dan interspSCNT telah dilaksanakan sebagai kajian awalan bagi penyelidikan SCNT kaprin. Kedua-dua klon embrio bovin dan gaur dapat dihasilkan secara *in vitro* sehingga tahap blastosis tetas dengan kadar perkembangan yang tidak berbeza secara signifikan (masing-masing 18.6% dan 19%).

Dalam Eksperimen 4, penambahbaikan penghasilan klon embrio kaprin *in vitro* dengan mempertimbangkan kesan tempoh pematangan, perlakuan pengaktifan dan protokol kultur *in vitro* telah dilaksanakan. Oosit kaprin daripada kambing betina yang disuperstimulasikan yang telah dimatang dalam tempoh 18 hingga 22 jam memberi

kadar pematangan, kadar enukleasi dan kadar perkembangan *in vitro* (IVD) (2-sel hingga tahap morula) yang lebih tinggi secara signifikan ($P<0.05$) selepas intraspSCNT berbanding oosit yang dimatang dalam tempoh 23 hingga 27 jam. Kedua-dua protokol pengaktifan [(7% EtOH + CD-CHX) dan (CaI + 6-DMAP)] mempunyai keberkesanan yang setanding dalam menginduksi perkembangan embrio rekonstruksi kaprin. Medium asas KSOMaa menampung perkembangan *in vitro* klon embrio kaprin lebih baik daripada mSOFaa dalam sistem kultur satu langkah di mana blastosis klon hanya dapat dihasilkan bila dikultur dalam KSOMaa seperti dalam kajian ini. Meningkatkan suplementasi glukosa kepada 2.78 mM dalam medium KSOMaa pada hari kedua kultur *in vitro* (IVC) meningkatkan kadar klon blastosis kaprin (19.9%) dan menggalakkan penetasan blastosis (15.6%).

Dalam Eksperimen 5, keberkesanan dalam penghasilan klon embrio kaprin menggunakan pendekatan SCNT intraspesies (intraspSCNT) berbanding interspesies (interspSCNT) telah dinilaikan. Kedua-dua pendekatan intraspSCNT dan interspSCNT membolehkan penghasilan klon blastosis kaprin dengan kadar masing-masing 17.3% dan 8.3%. Walau bagaimanapun, keberkesanan pendekatan interspSCNT adalah masih rendah berbanding pendekatan intraspSCNT. Tiada kebuntingan dikesan selepas percubaan pemindahan embrio ke atas klon embrio kaprin.

Secara ringkasnya, kajian ini telah menghasilkan beberapa penemuan yang baharu dalam senario penyelidikan reproduksi haiwan di Malaysia. Ini termasuk kejayaan dalam menghasilkan klon embrio kaprin sehingga tahap blastosis tetas melalui pendekatan intraspSCNT dan interspSCNT (karioplas kaprin- sitoplas bovin) menggunakan sel fibroblas kulit telinga kaprin; penemuaan satu sistem IVC baru yang dapat menampung perkembangan *in vitro* embrio kaprin ke tahap blastosis (menggunakan KSOMaa pada Hari 0 hingga Hari 2 dan meningkatkan suplementasi

glukosa hingga 2.78 mM dalam KSOMaa pada Hari 2 hingga Hari 8 kultur *in vitro*); protokol untuk intraspSCNT dan interspSCNT kaprin telah dibangunkan dan ini dapat digunakan sebagai rujukan untuk penyelidikan yang melibatkan pemindahan nukleus kambing dalam makmal ini pada masa hadapan.

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Sincerely,

Phek Jin

LIST OF PUBLICATIONS, PRESENTATIONS AND AWARDS

Parts of this study have been published in the following forms:

ISI Article

Kwong P.J., R.B. Abdullah and W.E. Wan Khadijah. 2012. Increasing glucose in KSOMaa basal medium on culture Day 2 improves *in vitro* development of cloned caprine blastocysts produced via intraspecies and interspecies somatic cell nuclear transfer. Theriogenology. 78: 921-929. (*ISI cited publication*)

Abdullah, R.B., W.E. Wan Khadijah and **P.J. Kwong**. 2011. Comparison of intra- and interspecies nuclear transfer techniques in the production of cloned caprine embryos. Small Ruminant Research. 98:196-200. (*ISI cited publication*)

Conferences (Oral Presentations)

Kwong, P.J., W.E Wan Khadijah and R.B. Abdullah. 2011. Production of cloned caprine blastocyst using intra- and interspecies SCNT approach. Proceeding of the 32nd Annual Conference of Malaysian Society of Animal Production (MSAP), June 6-9, Promenade Hotel, Tawau, Sabah, Malaysia. pp. 89-90 (Abstract).

Kwong, P.J., W.E. Wan Khadijah, R.B. Abdullah and R. Parnpai. 2010. Efficacy of cloned caprine embryos production using intraspecies- versus interspecies SCNT approach. Proceeding of the 31st Annual Conference of Malaysian Society of Animal Production (MSAP), June 6-8, Renaissance Hotel Kota Bharu, Kelantan, Malaysia. pp. 79-80 (Abstract).

Kwong, P.J., K. Sirattana, R. Parnpai, W.E. Wan Khadijah and R.B. Abdullah. 2009. *In vitro* production of cloned Gaur (*Bos gaurus*) blastocyst as an approach to conservation and domestication purposes. Proceedings of the 30th Annual Conference of Malaysian Society of Animal Production (MSAP), June 2-5, Hyatt Regency Hotel, Kota Kinabalu, Malaysia. pp. 43-44 (Abstract).

Conferences (Poster Presentations)

Abdullah R.B., **P.J. Kwong**, H.Y. Nam, W.E. Wan Khadijah and T. Kamarul. 2012. *In vitro* development of caprine embryos cloned with adult bone marrow mesenchymal stem cells. Proceeding of 9th Asian Reproductive Biotechnology Society (ARBS). October 23-27, Manila, Philippines. pp. 89 (Abstract).

Kwong, P.J., H.H. Soh, W.E Wan Khadijah and R.B. Abdullah. 2012. Effect of donor cell types on *in vitro* developmental potential of caprine interspecies somatic cell nuclear transfer embryos. Proceeding of the 11th International Conference on Goats (IGA). September 24-27, Gran Canaria, Spain. pp. 402 (Abstract).

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Abdullah, R.B., **P.J. Kwong** and W.E. Wan Khadijah. 2009. Production of cloned caprine embryos through interspecies somatic cell nuclear transfer approach. Proceeding of the 6th Annual conference of the Asian Reproductive Biotechnology Society (ARBS). November 16-20, Siem Reap, Cambodia. pp. 59 (Abstract).

Awards Obtained

Won the "Best Poster Presentation Awards" (Top three) in the 7th Asian Reproductive Biotechnology Society (ARBS) Annual Conference, November8-12, 2010, Kuala Lumpur, Malaysia.

Won the "Best Oral Presentation Award" in the 30th Annual Conference of Malaysian Society of Animal Production (MSAP), June 2-5, 2009, Hyatt Regency Hotel, Kota Kinabalu, Malaysia.

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%	percentage
^{o}C	degree celcius
β	beta
mHz	megahertz
µl	microlitre
ml	millilitre
µm	micrometer
mm	millimeter
cm	centimeter
µsec	microsecond
min	minute
hr	hour
mg	milligram
g	gram
mM	millimole
M	molar
mOsm	milliosmolarity
O_2	oxygen
CO_2	carbon dioxide
pH	hydrogen potential
kV/cm	kilovolt/centimeter
w/v	weight/volume
v/v	volume/volume
α-MEM	α-Minimum Essential Medium

6-DMAP	6-dimethylaminopurine
ABEL	Animal Biotechnology-Embryo Laboratory
ADP	adenosine diphosphate
AI	artificial insemination
ANOVA	analysis of variance
ART	advances reproductive technologies/ assisted reproductive technologies
ATP	adenosine triphosphate
BME	basal medium eagle
BSA-FV	bovine serum albumin-fraction v
CaI	calcium ionophore
CB	cytochalasin B
CBHA	m-Carboxycinnamic Acid Bishydroxamide
CC	cumulus cells
CD	cytochalasin D
CDK	cyclin dependent kinase
CHX	cycloheximide
CIDR	controlled intravaginal drug release device
CL	corpus luteum
COC	cumulus oocyte complexes
CR1	Charles Rosenkrans 1
CSF	cytostatic factor
CZB	Chatot, Ziomek, Bavister medium
DC	direct current
DMRT	Duncan’s Multiple Range Tests
DMSO	dimethyl sulfoxide

EDTA	ethylenediaminetetraacetic acid
EMiL	Embryo Micromanipulation Laboratory
ESC	embryonic stem cell
ESRC	Embryo Technology and Stem Cell Research Centre
ET	embryo transfer
eCG	equine chorionic gonadotrophin
e.g.	for example
et al.	et alii (and other)
FBS	foetal bovine serum
FCS	foetal calf serum
FSH	follicle stimulating hormone
G	gauge
H3-K9	lysine 9 on histone H3
ICI	intracytoplasmic injection
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
ID	inner diameter
IPPP	Institute of Research Management and Monitoring
IU	international unit
IVC	*in vitro* culture
IVD	*in vitro* developmental
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
IVP	*in vitro* production
intraspSCNT	intraspecies somatic cell nuclear transfer

interspSCNT	interspecies somatic cell nuclear transfer
i.e.	that is
KSOM	potassium simplex optimisation medium
KSOMaa	potassium simplex optimisation medium with amino acid
LH	luteinising hormone
LOPU	laparoscopic ovum pick-up
LOS	large offspring syndrome
M II	metaphase II
MAP	mitogen-activated protein
MEM	minimum essential medium
MPF	maturation promoting factor
mSOFaa	modified synthetic oviduct fluid with amino acids
mtDNA	mitochondria DNA
NaTuRe	Nuclear Transfer and Reprogramming Laboratory
NEBD	nuclear envelope breakdown
NT	nuclear transfer
OD	outer diameter
OGS	oestrus goat serum
OR	oocyte retrieval
oFSH	ovine-derived FSH
PA	parthenogenesis activation
P-S	penicillin-streptomycin
PB-1	first polar body
PBS	phosphate buffered saline
PMSG	pregnant mare’s serum gonadotrophin

PVP	polyvinylpyrrolidone
pFSH	porcine-derived follicle stimulating hormone
RO	reverse osmosis
SCNT	somatic cell nuclear transfer
SEM	standard error of means
SUZI	sub-zonal injection
TCM-199	tissue culture medium-199
TE	trophectoderm
TOC	total organic carbon
TSA	trichostatin A
TUGA	transvaginal ultrasound guided aspiration
TUNEL	terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling
UV	ultraviolet
Volt	voltage
vs.	versus
WCICI	whole cell intracytoplasmic injection
ZGA	zygotic genome activation

Chapter 1

1.0 INTRODUCTION

Chapter 1

1.0 INTRODUCTION

1.1 BACKGROUND

The application of assisted reproduction technologies (ART) in goat breeding program becomes a limelight in the 21st century. The yearly increasing demand of goat meat consumption and its dairy products worldwide, served as the moving gear in goat breeding industries to multiply the goat population via the application of ART besides sustaining the conventional breeding program. Among the ART that are applied in goat farming industry, reproductive cloning technology in production of cloned goat embryos is foreseen to facilitate the effort of mass goat production in just a short time frame.

Reproductive cloning, in particular somatic cell nuclear transfer (SCNT) is a laboratory technique used to create genetically identical embryos by replacing the genetic material of an oocyte (recipient cytoplast) with the genetic material of a somatic cell (donor karyoplast) from an adult organism to be cloned. SCNT can be conducted using two approaches either using recipient cytoplast and donor karyoplast of the same species, termed as intraspecies SCNT (intraspSCNT) or of different species termed as interspecies SCNT (interspSCNT). The commonly used somatic cell in SCNT includes mammary epithelial (Wilmut *et al.*, 1997), cumulus cells (Wakayama *et al*., 1998), foetal fibroblast cells (Cibelli *et al*., 1998) and skin fibroblast cells (Kato *et al*., 2000).

Generally, the SCNT procedure involves a series of steps such as enucleation, donor karyoplast transfer, activation, *in vitro* culture and embryo transfer. Either *in vitro* or *in vivo* matured oocytes could be used as the recipient cytoplast. Removal of the

genetic material of the matured recipient cytoplast (enucleation) is carried out using either squeezing or aspiration technique. Following enucleation of the oocyte, a somatic cell (donor karyoplast) is injected into the perivitelline space or directly into the cytoplasm of the enucleated oocyte (WCICI method) (Onishi *et al*., 2000). The enucleated oocyte and the donor cell in the perivitelline space are fused by electrofusion. After fusion of the donor nuclei and the enucleated oocyte, the reconstructed oocyte is activated by either chemical or mechanical stimulation. The activated reconstructed oocytes were subjected to *in vitro* culture, followed by transfer of the cloned embryos into a suitable recipient.

The SCNT technique offers several promises not only in the field of agriculture but also in the field of genetic conservation, medicine and basic cellular dynamic study. In the field of agriculture, particularly livestock industry, SCNT has the potential to impact animal breeding in as fundamental a manner as artificial insemination. Given its current high costs and relatively low success, SCNT will likely be used to improve production characteristics of food producing animals by providing breeding animals, just as any breeding program would select the most elite animals with desirable traits, (e.g. for milk containing extra nutrients or meat with more consistent in taste and quality) for breeding, and not as production animals. Cloning has the relative advantage of allowing for the propagation of animals with known phenotypes to serve as additional breeding animals. This is critically important in breeding programs, especially when it may take years to "prove" the merit of a sire or dam. Second, it allows the propagation of animals whose reproductive function may be impaired. Third, it allows the propagation of valuable deceased animals from which tissue samples have been appropriately collected or preserved, which may have profound implications for species or breeds nearing extinction. Finally, for the first time, cloning allows for the

careful study of the "nature-nurture" interactions that influence breeding programs by allowing a large enough sample of genetically identical animals to be raised in different environments, or with different diets. Such studies have been impossible to perform prior to the advent of SCNT and are likely to yield important information for developing livestock species to live in areas that have, until this time, been marginal for food animal production.

Conservation has been highlighted recently as an area where the SCNT technique may be useful. It may preserve and propagate endangered species that reproduce poorly in zoos until their habitats can be restored and populations reintroduced to the wild. Attempts have been made with the Giant Panda for instance. This technique allows maintenance or increase of the overall genetic diversity of a species by introducing new genes from preserved specimens or animals in other wild and captive populations of the same species back into a diminishing gene pool. SCNT, specifically interspecies SCNT (interspSCNT) may even recreate extinct species, if viable tissues or cells have been banked or are available. However, the work requires interspecies embryo transfer, as the surrogate female is usually a different but closely related species. The first endangered animal was born after cloned gaur interspSCNT embryos were transferred into a cattle that served as the surrogate mother (Lanza *et al*., 2000).

For the field of medicine, the greatest potential of the SCNT technique is in medical therapeutics in particular therapeutic cloning. Through SCNT approach, one's own embryonic stem cell (ESC) could be derived from the cloned preimplantation embryo produced. The ESC is useful in the treatment of degenerative diseases such as Parkinson's, AIDS, diabetes and muscular dystrophy.

SCNT can also be applied in xenotransplantation in which pig hearts, amongst other organs, engineered to lack the enzyme alpha-galactosyl transferase that creates proteins triggering hyperacute immune reactions may then be tolerated in human bodies. SCNT can also be used to grow autologous haematopoietic stem cells and bone marrow to replace blood-forming organs damaged by disease or radiation. Besides that it can be used in cancer treatment by cloning cells from cancerous tissue and introducing specific characteristics leading to early cell death (e.g. short telomeres). Reintroducing the altered cells could decrease the capacity for division and replication in the tumor.

In terms of the basic cellular dynamic study, SCNT served as a tool to study the nuclear reprogramming mechanism. When a nucleus from a differentiated somatic cell, is transplanted into an enucleated oocyte, nuclear reprogramming is initiated, leading to the generation of an entire individual, which is a genetically identical clone of the original somatic cell. Such nuclear-transfer experiments have shown definitively that all of the genes required to create an entire organism are present in the nucleus of the specialised cell and can be activated on exposure to reprogramming factors present in the oocyte. In other words, cell specialisation involves a change in gene expression, not in gene content, and the process of differentiation can be fully reversed (Yamanaka and Blau, 2010).

Up to date, various cloned animals were successfully produced such as sheep (Wilmut *et al*., 1997), cattle (Cibelli *et al*., 1998; Kato *et al*., 1998), goat (Baguisi *et al*., 1999), pig (Polejaeva *et al*., 2000), mouse (Wakayama *et al*., 1998) and monkey (Meng *et al*., 1997) via intraspecies SCNT (intraspSCNT). Extinct animals like gaur (Lanza *et al*., 2000), mouflon (Loi *et al*., 2001), bucardo (Folch *et al*., 2009) and banteng (Sansinena *et al*., 2005) were also successfully produced via interspSCNT approach. There were also several attempts to produce cloned animals via intergeneous SCNT

(Loi *et al*., 2011) however none of the cloned embryos successfully developed to term after embryo transfer. Even though live births were reported using both intraspSCNT and interspSCNT approaches, the overall efficiency is still low. To surmount this problem, there is still a wide scope of research which needs to be carried out to improve the technical and elucidate the biological factors influencing the success rate of SCNT.

1.2 STATEMENT OF PROBLEMS

Generally, the number of studies and the overall success rate of SCNT applied on caprine species is still low compared to other domestic animals like the bovine and ovine. Therefore there are still many areas of research that can be carried out to increase the success rate of caprine SCNT. Today, there are still several important questions pertinent to caprine SCNT such as:

1) How can we surmount the issue of shortage in caprine oocytes for countries that are deficient in obtaining caprine ovaries from abattoir?
2) If LOPU was employed in the caprine oocyte retrieval programme, which source of gonadotrophins or supertimulation regime can be used to increase the oocyte yield?
3) What is the meiotic competency and developmental fate of LOPU and abattoir-derived oocytes?
4) Do both the LOPU and abattoir –derived caprine oocytes matured optimally at the same IVM duration range?
5) Is the oocyte age (duration of MII arrest) affecting the developmental competency of the caprine reconstructed embryos?

6) Is the commonly used activation treatment for bovine SCNT such as 7% EtOH + CD-CHX could induce the development of caprine reconstructed oocytes and is the efficiency similar to the CaI + 6-DMAP activation treatments that are commonly used in caprine SCNT study?
7) What is the alternative IVC medium that can be used besides the common medium such as mSOFaa which is used in the current available caprine IVP study?
8) How can the blastocyst rate be improved in caprine SCNT and pertaining to this, will the improvement in IVC system influence the blastocyst production positively?
9) Last but not least is the current available caprine intraspSCNT protocols published could support the production of caprine intraspSCNT embryos similarly in all the laboratory setting?

1.3 JUSTIFICATION OF THE STUDY

Justification on the need and importance of this study was addressed by focusing on 4 issues. Firstly how intraspSCNT and interspSCNT can be beneficial to goat breeding industry? Why caprine is chosen as the experimental animal model? Why ear skin fibroblast cell is chosen as the donor nuclei or karyoplast? Why bovine oocyte is chosen as the recipient cytoplast for interspSCNT?

The SCNT either via intraspSCNT or interspSCNT approach could enable gender selection and production of elite goats with desirable traits, (e.g., for meat with consistent in leanness and taste as well as for milk production containing extra nutrients) in a more predictable manner compared to other ART involving fertilisation process by sperm. Farmers could develop the somatic cell line of the selected goats with known

phenotypes to be cloned in advance and cryopreserve the somatic cell lines for future usage. Cryopreservation of the somatic cell lines or cloned embryos provided convenience for propagation to be carried out without affected by the constraints of time and space. Furthermore, transgenic goats carrying genes that express pharmaceutical properties could be produced by SCNT approach through the usage of genetically modified somatic cell lines as donor karyoplasts. Besides that, the approach of interspSCNT allows the propagation of valuable deceased animals from which tissue samples have been appropriately collected or preserved, which may have profound implications for species or breeds nearing extinction such as serow goats.

Caprine was chosen as the model animal for this research due to the fact that it is a relatively convenient domestic species in terms of size and husbandry for current biological investigation and application. Its relatively short gestation period and its potential to produce diversified products of commercial value even justified further the importance of using caprine as the experimental model in this research; hoping that the findings of this study could be applied in the industry level. In Malaysia, goat meat is known to be an attractive choice for farming and production as it carries less taboo connotations unlike other types of meat and is more accessible in a multiracial and multi-religious country such as Malaysia. In addition, in a growing health conscious society, goat meat is steadily becoming a choice meat for many as it is has significantly lower fat (3%) compared to other meats such as cattle and sheep (16%). Besides that, goats are also known as the foster mother of man as their milk is considered better for human nutrition than milk of other species of livestock.

In this study, ear skin fibroblast cell is chosen as the donor karyoplast for the cloned caprine embryo production via intraspSCNT and interspSCNT due to the fact that adult skin fibroblasts cell are an easy-to-obtain source of donor DNA without the

limitations of animal age, sex, and physiological state. Thus, the gender of the cloned caprine produced can be selected and determined before development. In comparison to using other cell types such as cumulus, mammary epithelial, mural granulosa and oviductal cells in SCNT, the outcome are only limited to the production of female cloned embryos as these cell are only available from female donors (Wilmut *et al*., 1997; Wakayama *et al*., 1998). In addition, these cells are more difficult for long term culture compared to adult skin fibroblast cell.

As far as Malaysia is concerned, the possible main obstacle to produce cloned goat embryos by intraspSCNT approach is the paucity of goat ovary sources from abattoir, thus an alternative in using oocyte from closely-related species as recipient cytoplast are foreseen to overcome this hurdle. As bovine oocytes are relatively abundant in Malaysia and several studies have shown that ooplasm of bovine can support early development of embryos produced by interspSCNT of different mammalian species such as sheep, pigs, rats (Dominko *et al*., 1999) and gaur (Lanza *et al*., 2000), thus an experiment was carried out using bovine oocytes to produce cloned caprine embryos using interspSCNT approach in this research. Moreover, up to date there was no report available on caprine interspSCNT research using bovine as recipient cytoplast to generate cloned caprine embryos. Therefore, the findings of this experiment may contribute some new information on the research field of animal interspSCNT.

1.4 OBJECTIVES OF THE STUDY

The overall objective of this study was to produce cloned caprine embryos through intraspSCNT and interspSCNT techniques using caprine ear skin fibroblast cells as donor karyoplast. Since this study was pioneering the approach in producing cloned

caprine embryos in the local setting of Animal Biotechnology-Embryo Laboratory, University of Malaya as well as in the animal reproduction field of Malaysia, development of a SCNT protocol was also one of the major focus in this study. As a consequence, a series of experiments were designed to achieve this goal.

To achieve this goal, the following specific objectives were set to:

a) Study the effect of different sources of gonadotrophin (PMSG versus pFSH) on caprine superstimulatory responses.
b) Study the effect of different sources of caprine oocytes (LOPU- versus abattoir-derived ovaries) on the oocyte yield, grades and maturation performance.
c) Determine an optimised IVM duration range for caprine oocytes retrieved from LOPU- and abattoir- derived ovaries.
d) Develop cloned bovine and gaur embryos via intraspecies and interspecies SCNT approaches: a preliminary study for caprine SCNT research.
e) To study the effect of two different IVM intervals on cloned caprine IVD competency using ovarian-superstimulated caprine oocyte.
f) To study the effect of two different activation protocols on the IVD competency of reconstructed caprine embryos.
g) To study the effect of two different IVC media on the IVD competency of reconstructed caprine embryos.
h) To study the effect of increasing glucose concentration in KSOMaa medium at Day 2 culture on the IVD competency of reconstructed caprine embryos.
i) To compare the efficacy of producing cloned caprine embryos using intraspecies versus interspecies SCNT approaches.

Chapter 2

2.0 REVIEW OF LITERATURE

Chapter 2

2.0 REVIEW OF LITERATURE

2.1 BACKGROUND

Since 1930s, remarkable discoveries were obtained in the field of animal reproductive cloning. A dramatic research transition field from nature's ancient unassisted cloning to the modern era attempt to clone mammals in the laboratory has generated the discoveries of the possibility in producing viable cloned offspring through nuclear transfer technique. Nuclear transfer is not a new technique. It was first used in 1952 to study early development in frogs and in the 1980's the technique was used to clone cattle and sheep using cells taken directly from early embryos. In 1995, Ian Wilmut, Keith Campbell and colleagues created live lambs - Megan and Morag - from embryo derived cells that had been cultured in the laboratory for several weeks. This was the first time live animals had been derived from cultured cells and their success opened up the possibility of introducing much more precise genetic modifications into farm animals.

In 1997, Wilmut *et al.* (1997) in Roslin Institute and PPL Therapeutics announced their success in creating a cloned sheep named Dolly, it is the first animal cloned from a differentiated somatic cell taken from the mammary gland of an adult animal. The success of somatic cell nuclear transfer (SCNT) in cloning animal was further assured with the report by Wakayama *et al*. (1998) who successfully produced over 50 cloned mice using cumulus cell as donor karyoplast. Since then, the cloning of cattle, sheep, mice, goats and pigs have been reported (Table 2.1).

Table 2.1: Timeline of pioneering success in producing cloned animals of various species using SCNT approach

Year	Author	Species (cloned live birth)
1997	Wilmut *et al.*	Sheep (Dolly)
1998	Kato *et al.*	Cow
1998	Wakayama *et al.*	Mouse
1999	Baguisi *et al.*	Goat
2000	Polejaeva *et al.*	Pig
2000	Lanza *et al.*	Gaur
2002	Chesne *et al.*	Rabbit
2002	Shin *et al.*	Cat
2003	Zhou *et al.*	Rat
2003	Galli *et al.*	Horse
2004	Gomez *et al.*	Wild cat
2005	Lee *et al.*	Dog
2005	Sansinena *et al.*	Banteng
2005	Shi *et al.*	Swamp buffalo
2006(b)	Li *et al.*	Ferret
2007	Kim *et al.*	Gray wolf
2009	Folch *et al.*	Burcado

2.2 REGULATION OF REPRODUCTION IN FEMALE GOATS

The success in any animal *in vitro* production study could not be accomplished without the scientific understanding of the physiological process that occurs in the experimental animal on study. Since female goats were used as the donor for oocyte retrieval and as recipient for the embryo transfer experiments, oestrus synchronisation and superovulation protocols are required to control as well as to stimulate the reproductive events in the does. Thus, a brief review was made on the oestrous cycle, oestrus synchronisation and superovulation treatments of the doe in the following subsections.

2.2.1 Oestrous Cycle

The oestrous cycle refers to the recurring physiologic changes that are induced by reproductive hormones in most mammalian placental females. Oestrus was defined by McDonald (1980) as the sexual and fertile period of time which females of most mammalian will mate with males. The duration of oestrus was found to be varied between species. Edey (1983) reported that oestrus in sheep last for 30 hours while in goats, Evan and Maxwell (1987) found that the oestrus last for 25 to 30 hours. Chemineau *et al*. (1991) and Squires (2003) in their findings showed that the corpus luteum (CL) formed after luteinisation of the ovulating follicle is active where the secretion of the important amount of progesterone occurs during the luteal phase (14 days in the ewe and 16 days in the goats). Afterwards, luteolysis (CL regresses) occurs and a new cycle begins. Goats are known to be polyestrous as they have a number of cycles one after the other unless pregnancy occurs. The duration of the oestrous cycle depends on the development of the CL (Asdell, 1946) which is the dominant factor in the reproductive cycle in most species of the animals (John, 1971). The animals were said to have normal oestrous cycle when the oestrous cycle length was 21 ± 3 days

(Massita, 2003). Previous studies demonstrated that the duration of oestrous cycle can be influenced by factors such as, the age of the animal, breeds, stage of breeding season and environmental stress (Edey, 1983; Chibooka *et al*., 1988; Devendra and McLeroy, 1982; Jainudeen *et al*., 2000).

The duration of oestrous cycle for young animal was reported to be shorter and the period of intense oestrous was lesser compared to aged animals (Edey, 1983; Symington and Oliver, 1966). In goats, the oestrous cycle are classified as short (less than 17 days), normal (17 to 25 days) and long (more than 25 days) according to studies conducted by Gonzalez and Bury (1982), Thangavelu and Mukherjee (1982) and Massita (2003). In terms of breed factors, Katjang goats was reported to have an average length of oestrous cycle from 19 to 23 days (Thangavelu and Mukherjee, 1982); while for the Jermasia and Boer crossbred goats, the average oestrous length ranged between 19 to 22 days (Massita, 2003). Besides that, the oestrus duration of goat was observed to be shorter at the end of breeding season and in the first breeding season of young does. A summary on the characteristic events of oestrous cycle in doe are depicted in Table 2.2.

Table 2.2: Characteristics of oestrus in goats

Characteristic events	Average time
Duration of oestrous cycle	18-21 days
Duration of oestrus	24-48 hours
Time of ovulation	21-36 hours after oestrus

Source: Modified from Devendra and McLeroy (1982) and Jainudeen *et al*. (2000).

Generally, the periods of the oestrous cycle can be divided into four phase: prooestrus, oestrus, metaoestrus and dioestrus. These periods occur in a cyclic and sequential manner. In brief, proestrus refers to the period just before oestrus. It begins with the regression of the CL and drop in progesterone and extends to the start of the oestrus. One or several follicles of the ovary are starting to grow, their number being specific for the species. This phase can last as short as 4 to 5 days or as long as 3 weeks. Some animal may experience vaginal secretions, showing interest towards male goat but still refused sexually receptivity. Oestrus refers to the phase when female is sexually receptive (in heat). At this phase, ovarian follicles grow rapidly and maturing as well as the oestrogen secretions exerts their maximal influence. Metoestrus is a brief phase (3 to 5 days) that occurs with the cessation of oestrus or shortly after ovulation. During this phase the signs of oestrogen stimulation subside and the CL starts to form with multiple ovulations. Dioestrus is the longest phase in the oestrous cycle. This is a stage where the females refuse sexual activity with the male goats. During this period, the activity of CL functions fully, producing progesterone in which the size of CL decrease and ends with the regression of the CL.

2.2.2 Sign of Oestrus

During the oestrous cycle, each female will exhibit signs of oestrus for certain time. Sign of oestrus are indicators of onset of oestrus or heat and, therefore they are very important for oestrus detection. There two category of oestrus signs, namely the primary and secondary signs. The primary symptoms are the most reliable and most acceptable in indicating the oestrus behaviour in most animals. The best method of oestrus detection is by observing the primary symptoms exhibited by the female in response to the male. In goats, the primary symptoms that were usually portrayed including sign of

seeking out bucks, wagging of the tail when being exposed to the male, mounting behaviour and also bleating. This signs are obvious and generally more frequent if the male is absent (Chemineau, 1991). Besides that, physical signs such as redness and swelling of the vulva and a clear mucous discharge from the vulva also demonstrate that the female was in oestrus.

On the other hand, the secondary sign of oestrus that are used to detect does on oestrus including, restlessness, frequent urination, isolation and general loss of appetite and constant vocalisations. However, this signs are known to be less reliable because they vary in length and may confuse with the symptoms of a minor health problem. Although it is possible to detect oestrus signs in the doe as described above, but a doe in heat may not exhibit all the signs at the same time. These signs appear and disappear progressively with the onset and termination of oestrus behaviour. In order to obtain optimal results in oestrus detection, a routine check-up of heat twice daily (morning and evening) is indispensable (De Guzman, 1989).

2.2.3 Endocrinology Changes during Oestrous Cycle in Doe

Understanding on the endocrine patterns that occur during natural oestrus is important prior designing any oestrus synchronisation and supertimulation regime. The main events of the oestrous cycle are related to the period of growth of the ovarian follicles and the corpus luteum. Unlike the ewe, the information on hormonal changes during oestrous cycles in goat is sparse. According to Jainudeen *et al*. (2000), the sequence of hormonal events during the oestrus cycle is similar for both ewe and doe but the doe has longer progesterone phase than the ewe. Thus, due to the relatively similar hormonal events between ewe and doe, the hormonal patterns of LH, progesterone, oestradiol and

prostaglandin F_2 alpha ($PGF_{2\alpha}$) during the oestrous cycle of sheep was used as the reference to the endocrinology study of the doe (Figure 2.1).

During oestrus, the concentration of progesterone in blood is low (Pineda, 2003) and it remains low until Day 3 of early dioestrus. From Day 3 of dioestrus, progesterone levels will increase rapidly, reaching to maximal levels by Day 8 and remains stagnant until Days 11 to 12. By Day 13 of dioestrus, progesterone levels decline rapidly due to the abrupt regression of the CL caused by the lytic action of prostaglandin PGF2α which formed in the endometrium under the influence of progesterone and reaching the ovary by local route (Berisha and Schams, 2005) (Figure 2.1; [1]). This action subsequently causes the Graafian follicle to secrete significant amounts of androgen and oestrogen which reach peak values about Day 16 (Pineda, 2003) of the cycle (Figure 2.1; [2]). The rising oestrogen secretion triggers off a surge of LH released from the pituitary gland in episodic pulse after a time lag of about 12 hours (Figure 2.1; [3]). Oestrogen will induce the LH surge in the absence of progesterone. LH which enters the follicular fluid and acts on the target granulosa cells will cause ovulation, and follicular enlargement. The drop of progesterone level to less than 1 ng/ml and the rise of the oestrogen and androgen levels in the peripheral blood eventually bring the animal into behavioural oestrus (Figure 2.1; [4]). Ovulation occurs about 24 hours after the onset of the LH peak and the rises of the oestradiol in blood (Figure 2.1; [5]). Progesterone levels begin to rise as the CL develops and gradually acquires an increasing dependence on the constant low secreation of pituitary LH and prolactin for its maintenance and secretory activity. The high level of progesterone in the luteal phase prevents any sudden surge of LH release in response to the occasional waves of follicular development and oestrogen secretion that occur (Figure 2.1; [6]).

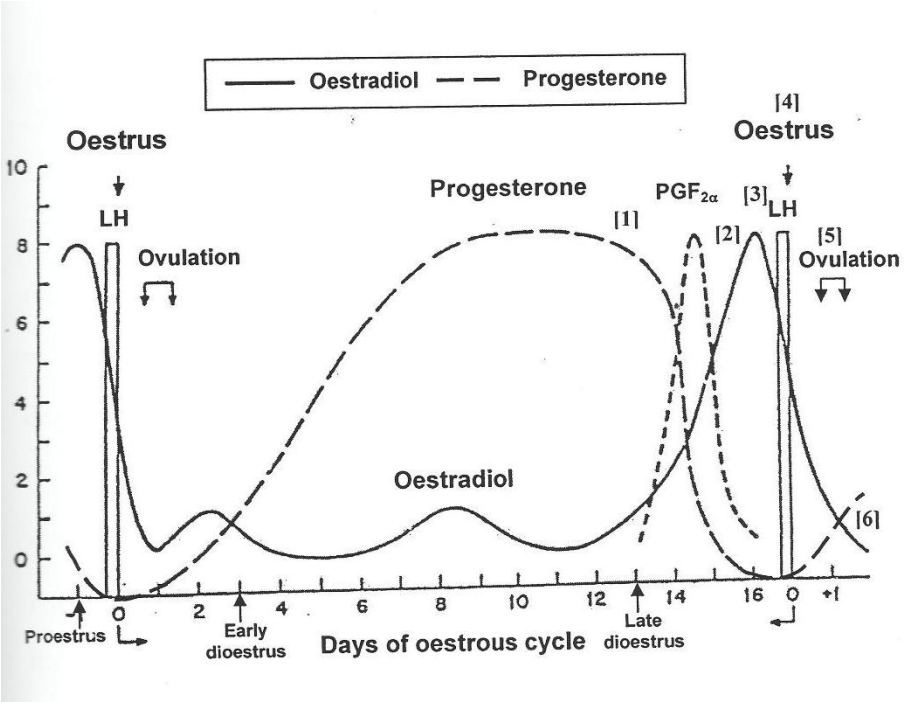


Figure 2.1: A model of the sheep oestrous cycle, indicating the sequence of hormone changes and the relationship between hormonal levels leading up to oestrus and ovulation. Description to key numbers are reflected in text (Adapted from Mohd Nadzir, 2006).

2.2.4 Follicular Dynamics

Ovarian follicular development during oestrus cycle was observed to occur in a wave-like pattern (Figueiredo *et al.*, 1997) and each wave being characterized by the synchronous development of a group of follicles (Baruselli *et al.*, 1997). The term of follicular wave is defined as one or more antral follicles growing from 3 mm to ≥ 5 mm in diameter before regression (De Castro *et al.*, 1999). The number of follicular waves that present during oestrous cycle varies among species. In cattle, 2 to 3 waves were detected while the growth of ovarian follicles in doe is characterised by the presence of 4 or more waves of follicle growth in the same cycle and it is in the final wave where the dominant follicle ovulates (De Castro *et al.*, 1999; Medan *et al.*, 2005). Unlike other

farm animals, the subsequent follicular wave begins even though the dominant follicle of the previous wave is still in its peak of development. This behaviour strongly suggests that follicular dominance is less apparent in the ovary of doe. Medan *et al*. (2005) also found that each follicular wave is preceded by an increase in FSH secretion.

2.2.5 Ovulation

Ovulation is defined as the rupture of the matured ovarian follicle on the surface of the ovary. The point of ovulation can be seen in the resulting corpus luteum for days after ovulation in most of the species. Ovulation is controlled by gonadotrophic hormones: FSH is predominant during the phase of follicular growth and LH is generally regarded as inducing ovulation and CL formation. In goat, LH was released from the pituitary in a surge (30 to 50 ng/ml) (Bono *et al*., 1983), which induces the final preparation of the follicle 24 hours prior ovulation phase. At ovulation in goats, the level of LH in blood reduces rapidly but FSH level begins to increase (Bono *et al*., 1983).

2.2.6 Oestrus Synchronisation

Oestrus sunchronisation is a hormonal treatment conducted to manipulation the response of female reproduction physiology in a timely manner. The control of oestrus and ovulation in farm animals remains the basis and a prerequisite for the success of controlled breeding. It is also a key element of all the ART-protocols in livestock and has a major influence to increase the overall efficiencies of these programmes (Baldassarre and Karatzas, 2004). Application of oestrus synchronisation plays an important role in fixing the time of breeding, AI, LOPU for oocyte or embryo collection and embryo transfer (ET). This technique has been developed since 1960s and this

treatment is important particularly for doe as the duration of both oestrous cycle and oestrus is variable; furthermore detection cannot be accomplished safely without a buck (Chemineau *et al.*, 1991).

Synchronisation of oestrus approaches in livestock generally focus on either manipulating luteal or the follicular phase of the oestrus cycle. Up to date there is a number of synchronisation methods developed for goats. The most widely used method in the doe is the treatment of progesterone or progestagen for 9 to 11 days followed by a luteolytic does of prostaglandin (or an analogue) administered in the period 36 to 48 hours prior to removal of intravaginal sponge (Baldassarre and Karatzas, 2004).

The progesterone treatment can be delivered through an intravaginal sponge, a CIDR (controlled internal drug release) device or alternatively using a subcutaneous implant (Evans and Maxwell, 1987; Frietas *et al*., 1997). Among the progesterone treatments mentioned, CIDR device is more commonly used. The CIDR device is developed in New Zealand and it is made of progesterone-impregnated medical silicone elastomers, with T-shaped vaginal insert containing 0.3 g of progesterone. The CIDR is inserted into the vagina of the treated females by a specialised applicator that collapses the wings for insertion. A thin nylon tail attached to the end of the CIDR is exteriorized through the vagina and is used to remove the device. CIDR delivers progesterone at controlled rate into the vagina and then into the bloodstream of the treated animals. After CIDR insertion, the presence of progesterone exerts a negative feedback effect on the hypothalamus, suppressing the release of two GnRH, that are, lutenising hormone (LH) releasing hormone and follicle stimulating hormone (FSH) releasing hormone which triggers the release of LH and FSH, respectively. This event prevents oestrus and ovulation. After removal of CIDR, the level of progesterone in the blood stream

declines which stimulates consequently a visible heat or oestrus behaviour (Massita, 2003).

2.2.7 Superovulation

Superovulation is a method for increasing the large number of ova released by the ovary induced with hormonal treatment. This method is important to accelerate genetic improvement in any species. Superovulation treatment could induce maturation, ovulation and to increase the number of follicles available for *in vivo* matured oocyte or *in vivo* produced embryo collection. While for superstimulation, usually the regime focus to just increase the number of follicles for the purpose of oocyte collection. Generally, the hormonal treatment for superstimulation and superovulation were quite similar. The principle of inducing superovulation in doe are similar as in cattle and ewe. An exogenous follicle-stimulating gonadotrophin is administered that mimics the effect of FSH the end of luteal phase of the cycle (days 9-11) or around 48 hours before FSH near the end of the synchronising treatments.

There are many ways to superovulate domestic animals, whereby each has its advantages and disadvantages. The major commercial products applied in most species are equine chorionic gonadotrophin (eCG, previously called PMSG) and FSH. Commercial preparations are partly purified from mare's serum and porcine pituitary glands, respectively. PMSG is used to stimulate ovarian activity during seasonal anoestrus and usually used concurrently following oestrus synchronisation. PMSG tend to be a practical hormone for superovulation due to its advantages of lower cost, most readily available, single dose protocol of up to 1500 IU to 2000 IU. However, the superovulatory response of PMSG can be quite variable and is usually lower than in FSH-induced superovulation (Amoah and Gelaye, 1990). PMSG-induced

superovulation is usually associated with problems like the present of high number of non-ovulated follicles, early regression of CL, short or irregular oestrous cycle and potential risk of embryo expulsion (Amoah and Gelaye, 1990). Thus a combination of eCG and hCG was widely used to superovulate does (Medan *et al*., 2003). On the other hand, FSH-induced does is known to give better ovarian responses and produced more oocyte than PMSG (Tsunada and Sugie, 1989; Pendelton *et al*., 1992). However, the requirement of multiple dosage injection when using FSH is rather labour-intensive and this poses a drawback of using FSH.

In order to surmount the cumbersome and the high expenses issue of using FSH, the attempts of using a 'one-shot' treatment regimen consisting of a single dose of FSH combined with the moderate dose of eCG (e.g., 60-80 mg FSH and 300 IU eCG) were reported (Batt *et al*., 1993; Baldassarre *et al*., 2002; Gibbons *et al*., 2007) and the outcome is almost equalled the oocyte or embryo yield with the traditional multiple injection regimen.

Besides the type of hormonal regimen influence the superovulatory response, both intrinsic and extrinsic factors are responsible for the variability as well. Intrinsic factors included genetic, age, stage of oestrous cycle during hormonal treatment (Wani *et al*., 1990; Mahmood *et al*., 1991) and extrinsic factors like season, nutrition and health state (Holtz, 2005) were identified to contribute to the variability in ovarian response. The timeline of significant finding in oestrus synchronisation and superovulation in does and ewes has been depicted in Table 2.3.

Table 2.3: Timeline of significant finding in oestrus synchronisation and superovulation in does and ewes

Year	Author	Significant finding
1983	Armstrong *et al.*	Stimulating an anoestrous doe with PMSG (400 IU) at the time or two days before removal of progestagen induced 2 to 3 follicles per doe.
1986	Greyling and Van Niekerk	Two injections of cloprostenol synthetic analogue at the rate of 62.5, 125.0 and 250 µg, administered 14 days apart, were effective in synchronising doe during breeding season.
1989	Hamra *et al.*	Using CIDR for 14 days and PMSG (750 IU) at withdrawal of subcutaneous implant, 92% oestrus response and 64% fertility were achieved.
1989	Tsunoda and Sugie	Higher numbers of oocytes were recovered with FSH-treated (9.4) than PMSG-treated (5.7) does.
1990	Amoah and Gelaye	Superovulation with PMSG are prone to premature regression of the induced CL, results in short cycles and have the potential risk of embryo expulsion.
1991	Chemineau *et al.*	Effective oestrus synchronisation was achieved using prostaglandin after Day 5 of oestrous cycle in does and ewes.
1994	Mani *et al.*	Good superovulation was achieved in Angora doe with porcine FSH (22 mg) divided in 4 decreasing dosage, injected twice daily, commenced one day before sponge removal after 17 days of progestagen treatment.
1994	Krisher *et al.*	Prostaglandin F_{2a} (PGF_{2a}) and GnRH is an effective supplement used with FSH superovulation regimens in dairy does which enhance early embryo collection for DNA microinjection studies.
1996	Freitas *et al.*	During anoestrus, fluorogestone acetate (FGA) intravaginal sponges for 11 days in conjunction with PMSG (750 IU) and cloprostenol (50 µg) 48 hours before sponge removal resulted in 87.5 and 93.8% oestrus response and fertility, respectively in Alpine does.

(continued)

Year	Author	Significant finding
1997	Mellado and Valdes	Found that ear implantation periods for both does and ewes usually extend from 9 to 14 days and often combined with PMSG and/or $PGF_{2\alpha}$ at 2 days before the end of ear implantation.
1997	Samsul	Does that have been synchronised with CIDR for 17 days together with PMSG 2 days before CIDR removal provided 100% oocyte recovery and 41.2% fertilisation rates, respectively.
1998	Muna *et al.*	Oestrus synchronisation in Sudanese Nubian does was achieved with double dose of cloprostenol (125 µg) together with intravaginal sponges impregnated with progesterone inserted for 16 days.
2000	Kusian *et al.*	Indicated that two intramuscular injections of cloprostenol (125 µg) administered 10 days apart in Mashana does were as effective as the progestagen treatments tested.
2001	Oliveira *et al.*	Oestrus synchronisation using CIDR for 9 days combined with eCG (100 IU) and cloprostenol at CIDR removal results a 100% oestrus in Saanen does within 24 hours.
2002	Motlomelo *et al.*	Concluded that the use of CIDR led to a shorter interval to onset of oestrus than 6-methyl-17-acetoxy-progesterone (MAP) and FGA.
2004	Romano	The use of FGA and MAP sponges and CIDRs with PG administration at the time of pessary removal induced efficient oestrus response and acceptable fertility in Nubian doe. The use of CIDR can be considered a worthy alternative to replace intravaginal sponges.
2006	Mohd Nadzir	Oestrus of mixed breeds of does was synchronised with CIDR for 10, 14, 17 or 21 days plus a single dose of 125 µg cloprostenol 36 hours before CIDR removal and superovulated with 70 mg FSH (Ovagen™) plus 1000 IU hCG (Profasi) 36 hours before LOPU resulted in recovery of 3.7- 6.7 oocytes per doe.

(continued)

Year	Author	Significant finding
2007	Cox and Alfaro	Oestrus of ewes and does were synchronised with CIDR-G® and superovulated with six injections of pFSH (Ovagen™) every 12 hours starting after 48 hours of CIDR-G insertion resulted in recovery of 14.6 oocytes per ewe and 21.9 oocytes per doe, respectively.
2007	Baldassarre *et al.*	Oestrus of aged Saanen and Toggenburg does was synchronised with MAP sponges for 10 days plus a single dose of 125 µg cloprostenol 36 hours before sponge removal and superovulated with 80 mg NIH-FSH-P1 (Follotrophin-V) plus 300 IU eCG 36 hours before LOPU resulted in recovery of 15.7 oocyte per doe.
2007	Gibbons *et al.*	Oestrus of ewes and does were synchronised with MAP sponges plus 50 µg PGF_{2a} and superovulated with 60 mg NIH-FSH-P1 (Folltrophin-V) plus 300 IU eCG 24 hours before LOPU resulted in recovery of 5.5- 8.8 oocytes per ewe and 5.6- 8.0 oocytes per doe, respectively.
2008	Melican and Gavin	Repeated superovulation and oestrus synchronisation in Saanen, Alpine and Toggenburg does with norgestomet implant or CIDR-G®, and non-surgical embryo retrieval, coupled with surgical embryo transfer, expedited the production of progeny from transgenic founder does.

2.3 CAPRINE OOCYTE RETRIEVAL

Generally, caprine oocyte can be collected from both live and dead does. Oocyte from live does can be collected using method such as laparotomy, laparoscopic ovum pick-up (LOPU) or transvagainal ultrasound-guided aspiration (TUGA). While oocytes from dead does can be obtained from the ovaries collected at post-mortem or from abattoir at slaughter. Ovaries from abattoir were subjected to either slicing or aspiration technique for oocyte retrieval.

Oocyte retrieval from slaughter does using aspiration method can be performed using needle of 18 to 22 G by puncturing and aspirating the follicular content. Besides that, oocyte retrieval can also be conducted using slicing method in which the surface of the ovaries was sliced in chequerbox manner followed by simple rinsing (Martino *et al.*, 1994). This approach was reported to produce more number of oocyte yield compared to aspiration method (Martino *et al.*, 1994; Pawshe *et al.*, 1996). Even though abattoir is an easy and cheapest source of oocyte worldwide, in Malaysia, this source is extremely limited due to low slaughtering activities as a consequence of shortage of breeding stock and when slaughtered, usually older or culled does are choosen (Rajikin, 1995). Thus OR from live does is an important alternative for embryo IVP study in Malaysia.

The oocyte recovery from live does can be accomplished with or without performing hormonal stimulation on the does prior retrieval. Oocyte retrieval via laparotomy is known to be invasive, causing adhesion of ovaries and other visera and other surgery related complication (Melican and Gavin, 2008). Thus, oocyte retrieval via LOPU and TUGA have been of preference as it is recognized as less traumatic than the standard laparotomy (Koeman *et al.*, 2003; Melican and Gavin, 2008). In comparison to LOPU, TUGA is more commonly used in large domestic animal and not much practice was reported in small ruminants like caprine. In a study conducted by

Graft *et al.* (1999), the number of follicles and oocytes retrieved from does using TUGA (9.5 and 4.3, respectively) was less than LOPU approach (17.4 and 14.4, respectively). Thus, at present, LOPU is still one of the best techniques for oocyte recovery from live does. LOPU is advantageous in which it allows repetition of the minor surgical retrieval procedure to be carried out more frequently and more oocyte retrieval (OR) cycle during the reproductive live of a valuable female (Baldassarre *et al*., 2007). LOPU usually resulted in > 5 oocytes aspirated per donor and it does not cause ovarian damage after several OR cycle (Alberio *et al*., 2002; Baldassarre and Karatzas, 2004). OR on does after multidose hormonal treatments via LOPU can be repeated at short intervals, even 4 days (Tervit *et al*., 1992).

2.4 *IN VITRO* MATURATION OF CAPRINE OOCYTES

The technology of *in vitro* production (IVP) of embryo is enhanced with the incorporation of the *in vitro* maturation (IVM) procedure. The IVM procedure enable mature oocytes to be generated ex vivo which involves the removal of cumulus oocyte complexes (COCs) from antral follicles of ovaries and culturing them in essential standard cell culture conditions until it reaches MII stage (Gilchrist and Thompson, 2007). The mature oocytes must be able to undergo both nuclear and ooplasmic maturation before they can be used for IVP of embryos.

2.4.1 Nuclear Maturation and Cytoplasmic Maturation

Both nuclear and cytoplasmic maturation are essential to ensure normal fertilisation and embryo development. When fully grown oocytes are removed from their follicles to be cultured *in vitro*, meiosis spontaneously resumed despite cytoplasmic maturity (Hyttel *et al*., 1997).

Nuclear maturation is reflected by the modification of the chromatin status from the dictyate phase (germinal vesicle) to the Metaphase II stage. Under *in vitro* condition, two major events are known to occur in order for the nuclear maturation to take place. First, the COC is removed from the influence of follicular environment and second, physical contact with mural granulosa cells is ruptured, terminating intercellular communication via the gap junctions. This chemico-physico stimulation of the oocyte causes condensation of the chromatin and breakdown of the GV leading to MII phase. At this point, a second artificial meiotic arrest is known to occur (Edwards, 1965).

On the other hand, cytoplasmic maturation encompasses all the changes in the distribution and organization of the individual organells from the GV to the Metaphase II stage. The cytoplasmic maturation includes the occurrence of stage-specific processes, such as the synthesis of specific proteins (Schultz and Kopf, 1995), the ability to release cortical granules, to release calcium from intracellular stores (Carroll *et al*., 1996), to re-localise mitochondria and to decondense sperm head (Thibault, 1977). Complete cytoplasmic maturation is essential as it is known to affect the subsequent developmental competency of the *in vitro*-derived zygotes (Eppig, 1996).

2.4.2 *In vitro* maturation (IVM) media

The ability of oocyte to undergo maturation *in vitro* requires appropriate dynamics within its microenvironment. A number of maturation media have been developed in different laboratory for IVM of goat oocytes. The general goat oocyte maturation medium contains TCM-199 supplemented with pyruvate, heat in activated serum, hormones and other additional supplementation (Ongeri *et al*., 2001; Izquierdo *et al*., 2002).

In most caprine IVM media, supplementation of gonadotrophins (FSH and LH) and oestradiol-17β are reported to improve maturation rates significantly (Keskintepe *et al.*, 1994; Izquierdo *et al*., 1999). The concentration of these hormones used varies among researchers. The range of concentration reported for FSH and LH was 0.1 to 10 µg/ml and 3 to 100 µg/ml, respectively (Cognié *et al*., 2003; Martino *et al*., 1995; Ongeri *et al*., 2001; Keskintepe *et al*., 1994). While for oestradiol-17β, the usual concentration is 1 µg/ml or some cases it is not supplemented at all in the IVM medium (Keskintepe *et al*., 1994; Pawshe *et al*., 1994b; Keefer *et al*., 2002). The supplementation of gonadotrophins in IVM medium was reported to enhance oocyte quality and development by possible alteration of metabolic processes. While for oestradiol, it is essential for cytoplasmic maturation in which it stimulates DNA polymerase β and enhanced the synthesis of presumed male pronucleus growth factors. The blastocyst production was reported to increase for oocytes matured in the presence of oestradiol-17β (Pawshe and Totey, 2003).

Supplementation of serum is usually included in IVM media because it is known to contain unidentified growth factors, hormones and peptides that may enhance the growth and development of oocytes. The source of serum that was used includes foetal

bovine serum (FBS), foetal calf serum (FCS), steer serum or oestrus goat serum (OGS) (Keskintepe *et al*., 1994; Crozet *et al*., 2000; Rodríguez-González *et al*., 2003).

Other supplementation like cysteamine or cystein, growth factors and vitamins was also included in IVM medium (Lott *et al*., 2011). Both cysteamine and cystein is a low molecular weight thiol that increases the intracytoplasmic oocyte glutathione (GSH) concentration and improves embryo development as well (De Matos and Furnus, 2000). GSH participates in various mechanisms such as amino acid transport, protein synthesis and protection against oxidative damage. Growth promoting factors like epidermal growth factor is also reported to enhance maturation of goat oocytes by triggering signal through MAPK pathway during IVM in goat cumulus cell. Bormann *et al.* (2003) also suggested that inclusion of vitamins into IVM medium increases the overall blastocyst development and mean blastocyst cell number.

2.4.3 *In Vitro* Maturation Duration

Earlier studies have reported a wide range of IVM duration required for the completion of meiosis in goat, ranging from 16 to 32 hours (Pawshe *et al*., 1994a; Crozet *et al*., 1995; Sharma *et al*., 1996). Differences in the reported IVM duration among researchers might be attributed to variations in the culture conditions and sources of oocytes.

Cognié *et al*. (2003) and Pawshe *et al*. (1994a) reported that caprine oocytes derived from stimulated ovaries generally started to mature as early as 16 hours after the initiation of maturation process and completed at 24 hours. Sharma *et al*. (1996) on the other hand demonstrated that oocytes collected from non-stimulated abattoir-derived ovaries achieve high maturation rate at 32 hours. Shorter IVM duration, 27 hours have

been reported for goat oocyte retrieved from ovaries procured from gonadotrophin treated goats (De Smedt *et al.*, 1992).

The IVM duration may affect not only the meiotic competency of the oocyte but it also influence the IVD of the oocytes after fertilisation or nuclear transfer. Prolonged MII arrest was reported to cause oocyte aging which leads to chromosomal anomalies and impair the development of embryos (Marston and Chang, 1964). It is thought that the oocyte aging process is related to changes in concentration of calcium ions (Yoon and Fissore, 2007), reactive oxygen species, activity of M-phase promoting factor (MPF) and mitogen-activated protein kinase (MAPK) (Miao *et al.*, 2009). A summary of significant findings in IVM for goat oocytes is depicted in Table 2.4.

Table 2.4: Timeline of significant findings in IVM of goat oocytes

Year	Author	Significant finding
1992	De Smedt *et al.*	Culturing abattoir COCs, 86% maturation rate was obtained from oocytes derived from 2- 6 mm sized follicles, whereas only 24% of oocytes from 1- 1.8 mm sized follicles were matured.
1995	Crozet *et al.*	Culturing COCs from superovulated and oestrus synchronised slaughtered doe ovaries in TCM-199 + FCS (10%) for 27 hours, 70, 83 and 97% maturation rates were obtained, respectively, from COCs derived from follicles of 2- 3 mm, 3.1- 5 mm and >5 mm in diameter.
1995	Martino *et al.*	Culturing abattoir-derived prepubertal goat COCs in TCM-199 + FBS (10%) for 27 hours, 72.0 and 76.9% maturation rates were obtained in presence or absence of GCs, respectively.
1996	Sharma *et al.*	Culturing abattoir-derived COCs in TCM-199 + OGS (20%), 71.6, 59.7, 55.8 and 50.3% maturation rates were obtained respectively, after 32, 36, 31 and 24 hours of IVM.

(continued)

Year	Author	Significant finding
1996	Gall *et al.*	Culturing COCs from hormonally stimulated slaughtered goat ovaries in TCM-199 + FCS (10%), 22 and 96% maturation rates were obtained, respectively, after 20 and 27 hours of IVM of COCs derived from 2- 6 mm sized follicles. However, only 3.5 and 8% maturation rates were obtained with COCs from 1- 1.8 mm sized follicles after 20 an 27 hours of IVM.
1999	Malik *et al.*	Culturing abattoir-derived COCs for 28 hours, 63.6, 55.6 and 44.6% maturation rates were obtained, respectively, in TCM-199 + OGS (20%), goat peritoneal fluid and rabbit peritoneal fluid media.
2000	Samaké *et al.*	Culturing COCs from superovulated and oestrus synchronised does in TCM-199 + FBS (10%) for 24 hours, 100% maturation rates were obtained from COCs retrieved by both laparotomy and ovariectomy methods.
2001	Rho *et al.*	Culturing abattoir-derived COCs in M-199 + FCS (10%), 73, 55 and 30% maturation rates were obtained, respectively, after 27, 24 and 20 hours of IVM.
2002	Cognié *et al.*	Culturing goat oocytes in TCM-199 + FF (10%) with (+) or without (-) cysteamine (100 µM), maturation rates of 21% (-) and 32% (+); 46% (-) and 71% (+); and 72% (-) and 79% (+) were obtained, respectively, after culturing for 16, 20 and 24 hours.
2003	Rodriguez-Gonzalez *et al.*	Culturing abattoir-derived prepubertal goat COCs in TCM-199 + SS (10%) supplemented with 100 µM cysteamine for 27 hours, significantly higher maturation rate was obtained from brilliant cresyl blue (BCB) positive (Blue colour) COCs (89.5%) than BCB negative COCs (72.1%) or unselected (control) COCs (67.3%).
2005	Nagar and Purohit	Culturing abattoir-derived COCs with different concentration of EGF in TCM-199 medium for 28 hours, 34.1, 55.6, 64.5, 52.4, and 49.2% maturation rates were obtained, respectively, with supplementation of 0, 10, 20, 50 and 100 ng/ml EGF.

(continued)

Year	Author	Significant finding
2006 2007	Phua; Amir	Culturing LOPU-derived oocytes from oestrus synchronised and superovulated does in TCM-199 + OGS (10%), 31.0- 67.8% maturation rates were obtained after 18 to 24 hours of IVM.
2008	Abdullah *et al.*	Oocytes yield after a prolonged interval between hormonal stimulation and LOPU (72 hours) achieve maturation rate of 82%.
2010	Kwong *et al*.	Oocytes yield after a prolonged interval between hormonal stimulation and LOPU (66- 72 hours) achieve maturation rate of 75% after subjected to IVM duration of 18- 22 hours.

2.5 SOMATIC CELL NUCLEAR TRANSFER (SCNT)

Somatic cell nuclear transfer (SCNT) cloning involves removing nuclear DNA from a mature oocyte (enucleation) and inserting a donor cell nucleus (reconstruction) derived from a somatic cell. The donor nucleus is then subjected to a complete "reprogramming" by undetermined factors located inside the ooplasm, which enable the complete set of instructions that were once turned off in the differentiated donor nucleus to become active and commence development, not as another somatic cell, but as a 1-cell embryo (Campbell, 1999).

2.5.1 Manipulation Technique

Each group of researchers has its own specific technique in producing cloned animal. There are three manipulation techniques successfully developed for SCNT namely, Roslin technique, Honolulu technique and handmade cloning. The former two

approaches were widely used and both share the same concept as the somatic donor karyoplast used were in G0 stage.

Roslin Technique was developed in the Roslin Institute, by Ian Wilmut, Keith Campbell and fellow researchers. This approach involve the enucleation of a matured sheep oocytes by squeezing approach and subsequently a cell cycle- synchronised donor cell (mammary gland cell) by serum starvation was injected into the perivitelline space of the oocyte. An electrical pulse was used to fuse the donor cell with the oocyte cytoplast, at the same time activate the development of the reconstructed oocyte. In their study, the cloned sheep embryos were cultured in sheep's oviduct and subsequently transferred into the uterus of the surrogate mother. The first successful delivered cloned sheep name Dolly was produced using this approach (Wilmut *et al*., 1997).

Honalulu technique was developed in the University of Hawaii by Teruhiko Wakayama and Ryuzo Yanagimachi. In this manipulation method, enucleation was carried out using aspiration technique and the donor cell or karyoplast (cumulus cell) were injected into the cytoplasm of the oocytes after breaking the cell membrane of the donor cell. The reconstructed oocyte was then subjected to chemical activation treatment prior *in vitro* culture to develop cloned embryos. Using this approach, they had successfully produced cloned mice (Wakayama *et al*., 1998). Both Roslin and Honalulu techniques required delicate instrumentation, special manual skills and hard, concentrated work. Ever since both these technique were successfully used to cloned sheep and mice, other researcher using both approaches had successfully produced cloned cattle, goat and pig (Table 2.1)

Handmade cloning is an alternative method in which it does not involve the use of micromanipulator and reconstructed embryos were leave to develop without zona pellucida. The only successful published attempt to make cloning without manipulation

was the bovine system established by Peura *et al*. (1998, 2001) based on removal of the zona pellucida, oriented enucleation with a handheld blade under a stereomicroscope, and fusion with a blastomer by using the AC current in the fusion chamber to keep cells together. The overall efficiency of this system was commeasurable with that of traditional cloning, but initial attempts to apply the procedure for somatic cell nuclear transfer were unsuccessful (Trounson *et al*., 1998). Later the improved handmade cloning technique was developed for somatic cell nuclear transfer (Vajta *et al*., 2001). Handmade cloned offspring have been produced in cattle (Tecirlioglu *et al*., 2003; Vajta *et al*., 2003), horse (Lagutina *et al*., 2005), pig (Du *et al*., 2005) and buffalo (George *et al*., 2011).

2.5.2 SCNT Approach

2.5.2.1 Intraspecies SCNT

Intraspecies SCNT (intraspSCNT) approach involves the transplantation of a donor cell (karyoplast) into a recipient enucleated oocyte (cytoplast) of the same species. Generally the success rate of intraspSCNT approach is higher compared to interspSCNT approach and this might be attributed to the fact that the phylogenetic distance between the animals in which the oocyte and somatic cell was derived in intraspSCNT approach is closely related. In the intraspSCNT approach, the efficacy was further influenced by the application of autologous and heterologous approach.

Autologous SCNT refer to the method in which somatic nucleus of a female donor is transferred to its own enucleated oocyte (Yang *et al*., 2006), while heterologous approach involve the transfer of a donor cell into an enucleated oocyte of the unrelated individual of the same species. Using the autologous approach, the

molecular and genetic between the nucleus and cytoplasm of the cloned embryos could be preserved. On a more cellular level, the interaction between the donor nucleus and recipient cytoplast may influence a number of important biological functions in SCNT during nuclear reprogramming. When considering the karyoplast-cytoplast interaction, mitochondria are the most abundant organelle in cytoplasm and play an important role in development by supplying energy for normal cellular functions. Mitochondrial DNA (mtDNA) is supplied mainly by the recipient oocyte during SCNT, but is regulated by genes in the donor nucleus. Under the high oxygen environment, and with limited DNA repair ability, mtDNA has high rates of heritable polymorphism and de novo mutation which can result in many haplotypes. In cattle, oocytes with various mtDNA haplotypes usually have different ATP content and this may affect the efficiency of *in vitro* production of embryos (Bruggerhoff, 2002; Yan *et al*., 2010). By using autologous SCNT approach, this hurdle could be solved.

In caprine SCNT study, the first cloned kid was produced in the year 1999 by using intraspSCNT approach. Even though live cloned kids were produced, the overall efficiency is still low. Subsequent studies were carried out by various laboratories to improve the caprine intraspSCNT efficiency as depicted in Table 2.5.

Table 2.5: Timeline of significant findings intraspSCNT in caprine

Year	Author	Significant event/ finding
1999	Baguisi *et al*.	First report on goat SCNT live birth in which 3 female offsprings were produced using foetal somatic cells as donor karyoplast.
2001	Keefer *et al*.	Both *in vitro* transfected caprine foetal fibroblast cells could direct full term development following nuclear transfer.

(continued)

Year	Author	Significant event/ finding
2001	Reggio *et al*.	First report of cloned goats produced from nuclear transfer using cytoplasts derived from abattoir ovaries.
2001	Zou *et al.*	The survival rate of cloned-caprine embryos obtained by injection was higher than that derived from fusion (62.7 and 45.9%).
2002	Guo *et al*.	The results of microsatellite DNA analyses indicated that the 2 kids were from the same donor fibroblast cell line derived from an adult caprine ear skin.
2002	Keefer *et al*.	No significant differences in the rates of pregnancy and nuclear transfer efficiency between granulosa cells and foetal fibroblast cells.
2002	Zou *et al.*	A foreign gene, such as the neo-resistant gene could be introduced into caprine foetal fibroblast cells and that the resulting transgenic cells were capable of being cloned to produce 100% transgenic animals.
2003	Das *et al*.	Reported that 300 V resulted in better electrofusion and cytochalasin B blocked synchronised cells and fast growing skin fibroblast cells of caprine could be used for nuclear transfer.
2003	Ohkoshi *et al*.	Caprine nuclear transfer using anterior pituitary cells in an *in vitro* culture system had the developmental potential to produce offspring after embryo transfer.
2004	Zhang *et al*.	Donor cell cycle at stage G0/G1 might be efficient ways to improve the developmental competence of reconstituted caprine embryos than stage G2/M.
2005	Melican *et al*.	More offspring were produced utilising karyoplast cultured in low serum versus cycling cells grown to confluence to synchronise G0/G1 stage cells. In addition, more live offspring were produced using donor cells harvested by partial compared with complete trypsinisation.

(continued)

Year	Author	Significant event/ finding
2006	Lan *et al*.	The fusibility and *in vitro* developmental potential of embryos reconstructed from foetal fibroblasts at passages 20 to 25 were significantly lower than those of embryos reconstructed from foetal fibroblasts at passages 3 to 5 and the cloning efficiency of the long term cultured cells was low (0.5%).
2006	Shen *et al*.	SCNT is a viable technique for goat cloning and that increase electrical field strength for both fusion and activation of reconstructed embryos appeared to be beneficial for the development of cloned embryos.
2007	Chen *et al*.	The method of telophase II enucleation combined with whole cell intracytoplasmic injection (WCICI) could properly reprogramme the somatic cells, and WCICI could provide an efficient and less labour-intensive protocol in Asian yellow goat cloning.
2008	Daniel *et al*.	The difference in membrane surface properties between cumulus and fibroblast cell may contribute to the higher fusion rate obtained in cumulus cells for cloned caprine embryo production.
2008	Tao *et al*.	Both interspecies and intraspecies reconstructed cloned-caprine embryos have similar development changes in the zona pellucida, rough endoplasmic reticulum, Golgi apparatus and nucleolus when compared with *in vivo* produced embryos.
2008	Wang *et al.*	Successfully developed an effective method to screen transgenic donor cells and improve the production efficiency of transgenic embryos.
2009	Tao *et al*.	Optimised nuclear transfer protocol and proper hCG treatment led to the successful birth of a cloned goat.
2009	Yuan *et al*.	Live goats were generated by SCNT from caprine mammary gland epithelial cells using long term cultured cell lines (25 to 27 passages).

(continued)

Year	Author	Significant event/ finding
2010	Akshey *et al*.	The foetal fibroblast cell was a suitable candidate as nuclear donor and the flat surface culture system was suitable for zone-free blastocyst development by the hand-made cloning technique in the goat.
2010	Zhang *et al*.	Successfully produced functional expression of hGCase in mammary gland cells and normal development to Day 40 of cloned embryos carrying the hGCase gene.
2010	Dalman *et al*.	The use of full confluency was suitable for cell cycle synchronisation because it arrested cells at the G0/G1 phase and also induced less apoptosis in comparison with the serum starvation group.
2011	Abdullah *et al*.	Cloned-caprine embryos could be produced *in vitro* via both intraspSCNT and interspSCNT approaches in which the efficacy of interspSCNT approach was comparable to that of intraspSCNT approach.
2011	Akshey *et al*.	First report of effect of treatment of donor cells with roscovitine and different activation methods on handmade cloned embryo production in goat.
2011	Liu *et al*.	CB treatment for 2 to 3 h between fusion and activation was an efficient method for generating cloned goats by SCNT. In addition, increasing the number of embryos transferred to each recipient resulted in more live offspring from fewer recipients.
2011	Nasr-Esfahani *et al*.	First report of successful live and survived birth of cloned and transgenic kids using zona-free method of SCNT.
2011	Tang *et al*.	mSOF medium supplemented with 10% FBS could better support the development of cloned caprine embryos and he blastocysts cultured in this medium could develop to term and gave birth to a healthy kid at term.

2.5.2.2 Interspecies SCNT

Interspecies SCNT (interspSCNT) approach involves transplantation of a donor cell (karyoplast) into a recipient enucleated oocyte (cytoplast) of a different species/family/order /class. The interspSCNT approach would be beneficial in any situation, in which an alternative source of ooplasm is needed, due to either ethical or technical considerations, such as establishing primate ESCs from interspSCNT embryos or cloning endangered species, respectively. The ultimate endpoints of these applications are either (1) to generate a preimplantation embryo to be used as a source of ESCs or (2) to produce live offspring in all animals with the exception of human.

Successful interspSCNT, either giving birth to live offspring or producing nearly termed fetuses, has been achieved by several groups concerned with the conservation of endangered species. The first interspSCNT offspring was reported for the gaur by Lanza *et al*. (2000) but unfortunately died within the first 48 hours. Using domestic sheep (*Ovis aries*) as recipient cytoplasts, two pregnancies were established after interspSCNT using an exotic argali (*Ovis ammon*) for donor karyoplasts, however both of these pregnancies were reported to have been lost by 59 days of gestation (White *et al*., 1999). More recently, domestic sheep (*Ovis aries*) enucleated oocytes used as cytoplasts for adult mouflon cells (*Ovis orientalis musimon*) resulted in one live offspring (Loi *et al*., 2001).

The animal species in which their oocytes were reported to be used as the recipient cytoplast for the interspSCNT studies included cattle (Dominko *et al*., 1999; Lanza *et al*., 2000; Yoon *et al*., 2001), rabbit (Chen *et al*., 2002; Wen *et al*., 2005), pig (Ikumi *et al*., 2004; Hashem *et al*., 2007), goat (Folch *et al*., 2009), buffalo (Lu *et al*., 2005; Yang *et al*., 2010), sheep (Loi *et al*., 2001), domestic cat (Gomez *et al*., 2004) and dog (Kim *et al*., 2007). While the type of somatic cells commonly employed in

interspSCNT study included dermal fibroblasts (Lanza *et al*., 2000), granulosa cells (Loi *et al*., 2001) and foetal fibroblasts (Kitiyanant *et al*., 2001) which resulted in either offspring or blastocyst production.

Even though, cloned offspring were able to be produced using interspSCNT approach, the overall efficiency of cloning is low in all species owing to not only the incomplete nuclear reprogramming of differentiated cells but also the factors of mitochondria/ genomic DNA compatibility, embryonic genome activation of the donor nucleus by the recipient oocyte and the availability of suitable foster mothers for interspSCNT embryos (Loi *et al*., 2011).

Since this study involve the production of caprine interspSCNT embryos using bovine cytoplast, a summary of interspecies cloning using ruminant oocyte as recipient cytoplast was presented in Table 2.6 for reference.

Table 2.6: Summary of interspecies cloning using ruminant oocyte as recipient cytoplast

Taxonomic relationship	Recipient cytoplast species	Donor karyoplast Species (**Cloned species**)	Type of donor karyoplast	Significant development	References
Intersubspecies (intraspecies)	Goat (Saanen)	Goat (Boer)	Ear skin fibroblast cell	Offspring	Jian-Quan *et al*., 2007
	Swamp buffalo (*Bubalus bubalis carabensis*)	River buffalo (*Bubalus bubalis bubalis*)	Ear skin fibroblast cells	Offspring	Yang *et al*., 2010
Interspecies (intrageneric)	Cattle (*Bos taurus*)	Gaur (*Bos gaurus*)	Skin fibroblast cells	Offspring	Lanza *et al*., 2000
		Yak (*Bos grunniens*)	Cumulus cells	Blastocyst: 10.9%	Murakami *et al*., 2005
			Ear skin fibroblast cell	Blastocyst: 28.0%	Li *et al*., 2007
		Banteng (*Bos javanicus*)	Ear skin fibroblast cells	Blastocyst: 15– 28%	Sansinena *et al*., 2005
	Goat (*Capra hirus*)	Burcado (*Capra pyrenaica pyrenaica*)	Ear skin fibroblast cells	Offspring	Folch *et al*., 2009
	Sheep (*Ovis aries*)	Argala (*Ovis ammon*)	Skin fibroblast cell	Foetuses: 50 days old	White *et al*., 1999
		European Mouflon (*Ovis orientalis musimon*)	Granulosa cell	Offspring	Loi *et al*., 2001

(continued)

Taxonomic	Recipient cytoplast species	Donor karyoplast species (**Cloned species**)	Type of donor karyoplast	Significant development	References
Intergeneric (intrafamily)	Cattle (*Bos taurus*)	Buffalo (*Bubalus bubalus*)	Foetal fibroblast cells	Blastocyst: 33.0%	Kitiyanant *et al*., 2001
			Ear skin fibroblast cells	Blastocyst: 4.5%	Lu *et al*., 2005
		Sheep (*Ovis aries*)	Ear skin fibroblast cells	Blastocyst: 18.0%	Dominko *et al*., 1999
				Blastocyst: 24.0%	Hua *et al*., 2008
		Goat (*Capra hircus*)	Foetal fibroblast cells	Blastocyst: 7.9%	Song *et al*., 2008
		African buffalo (*Syncerus caffer*)	Ear skin fibroblast cells	Blastocyst: 3.5%	Matshikiza *et al*., 2004
		Eland (*Taurotragus oryx*)		Blastocyst: 2.0%	
		Takin (*Burdocas taxicolor*)	Ear skin fibroblast cells	Blastocyst: 5.0%	Li *et al*., 2006a

(continued)

Taxonomic relationship	Recipient cytoplast species	Donor karyoplast Species (**Cloned species**)	Type of donor karyoplast	Significant development	References
	Buffalo (*Bubalus bubalus*)	Cattle (*Bos taurus*)	Ear skin fibroblast cells	Blastocyst: 3.0%	Lu *et al*., 2005
	Sheep (*Ovis aries*)	Goat (*Capra hircus*)	Foetal fibroblast cells	Blastocyst: 7.4%	Ma *et al*., 2008
Interfamily (intraorder)	Cattle (*Bos taurus*)	Pig (*Sus scrofa*)	Ear skin fibroblast cells	Blastocyst: 6.0%	Dominko *et al*., 1999
			Skin fibroblast cells	Blastocyst: 3.9%	Uhm *et al*., 2007
	Sheep (*Ovis aries*)	Camel (*Camelus bactriancus*)	Ear skin fibroblast cells	Blastocyst: 0%	Zhou and Guo, 2006
Interorder (intraclass)	Cattle (*Bos taurus*)	Rhesus monkey (*Macaca mulatta*)	Ear skin fibroblast cells	Blastocyst: 16.6%	Dominko *et al*., 1999
		Crab-eating monkey (*Maccaca fascicularis*)	Skin fibroblast cells	16-cell: Not Available	Lorthongpanich *et al*., 2008
		Mouse (*Mus musculus*)	Embryonic fibroblast cells	8-cell: 6.2%	Arat *et al*., 2003
	Goat (*Capra hirus*)	Human (*Homo sapiens recens*)	Neural stem cells	Blastocyst: 10.7%	Sha *et al*., 2009

(continued)

Taxonomic relationship	Recipient cytoplast species	Donor karyoplast Species (**Cloned species**)	Type of donor karyoplast	Significant development	References
Interclass	Cattle (*Bos taurus*)	Fowl (*Gallus gallus domesticus*)	Embryonic fibroblast cells	Blastocyst: 3.0%	Kim *et al*., 2004

2.6 FACTORS AFFECTING NUCLEAR TRANSFER.

2.6.1 Micromanipulation

Nuclear transfer is a complex procedure, which involve the micromanipulation procedure such as enucleation and donor karyoplast transfer prior activation, *in vitro* culture and embryo transfer. Each of these steps affects the overall efficiency of the cloning outcome.

2.6.1.1 Enucleation

Prior to nuclear transfer (NT), the recipient oocyte DNA has to be removed or destroyed without compromising viability and reprogramming potential of the cytoplast. Enucleation can be accomplished by using solely mechanical approach or with chemical-assisted enucleation approach and centrifugal enucleation.

The solely mechanical approach is usually carried out using squeezing approach. Presuming that the MII plate located adjacent to the first polar body (PB-1), a slit is made in the zona pellucida above PB-1 with a microneedle and subsequently by applying mild pressure, PB-1 and a small amount of cytoplasm assumed containing MII plate was squeezed out (Tsunoda *et al.*, 1989). The squeezed out material is subjected to Hoechst 33342 dye to confirm positive enucleation. This technique is also known as 'blind squeezing method'. By using this enucleation method, the oocyte can be avoided from the harmful effects of ultraviolet (UV) light. The detrimental effects of DNA dyes and ultraviolet light exposure on oocytes and their developmental potential have been documented in the past (Smith *et al.*, 1993; Dominko *et al.*, 2000). Although this technique eliminates the use of UV light, it has been documented that first polar bodies

of a metaphase-II oocyte often migrate and do not always remain in proximity to the chromosomes in the metaphase-II spindle (Bordignon and Smith, 1998; Dominko *et al*., 2000; Liu *et al*., 2000).

An alternative to the 'blind squeezing' enucleation approach is the chemically assisted enucleation. Fluorochrome labeled enucleation was accomplished by labeling the matured oocyte DNA with fluorochrome (Hoechst 33342) prior enucleation (Critser and First, 1986). The labeled oocyte was subjected to UV irradiation during enucleation to locate the MII spindle. Fluorochrome labeled enucleation was reported to produce higher enucleation efficiency (Westhusin *et al*., 1992; Smith, 1993), however when these oocyte were exposed to UV light for > 30 seconds, it was reported to cause a loss in membrane integrity, decreases methionine incorporation, alters protein synthesis patterns in the oocytes (Smith, 1993). Pretreatment of oocytes with 3% sucrose was also effective in aiding the visualisation of the metaphase spindle and chromosomes with standard light microscopy (Wang *et al*., 2001) and a 0.3 M sucrose treatment was found to be effective in facilitating the localisation of the mouse and bovine chromosomes (Liu *et al*., 2002b). Demecolcine is another chemical that could be used to assist in enucleation of oocytes by causing membrane protrusion that contained the condensed chromosomes, which were easily visualised and aspirated into a pipette without the need of staining (Yin *et al*., 2002). Other chemically-induced enucleation method includes using etoposide (Elsheikh *et al*., 1998), etoposide in conjunction with cycloheximide (Fulka and Moor, 1993), and ethanol with demecolcine (Ibanez *et al*., 2003). While these methods greatly facilitate the enucleation process, development of NT embryos following chemically-induced enucleation remains lower than development of mechanically enucleated oocytes (Gasparrini *et al*., 2003).

Because the enucleation step requires a high degree of visual and manual control, other methods of enucleation have also been explored such as centrifugal enucleation and zona-free with manual bisection approach. The centrifugal enucleation method takes advantage of the fact that the genetic material is heavier than the cytoplasm. If zona-free oocytes are centrifuged in an appropriate gradient in the presence of a cytoskeletal inhibitor, a karyoplast containing the metaphase plate can be separated from the cytoplasm (Wagoner *et al.*, 1996). While for the zona-free with manual bisection approach, the MII oocytes were stripped of their zonae pellucidae using pronase and manually bisected using an Ultra Sharp splitting blade (AB Technologies, Pullman, WA). The demi-oocytes were then stained for nuclear DNA and only those without a metaphase spindle were selected for fusion with donor somatic cells (Oback *et al*., 2003; Vajta *et al*., 2003).

As for species with fragile oocytes or hard zona pellucida, such as the mouse and the horse, Piezo-driven enucleation and reconstruction (Wakayama *et al*., 1998; Choi *et al*., 2002b) are usually incorporated. This is because the Piezo drill generates mechanical pulses that travel longitudinally along the microinjection pipette and vibrate the pipette tip, drilling through the zona pellucida and the oolema are facilitated without producing any net forward movement of the pipette.

The success of removing the nuclear material of the matured oocytes without causing damage to the cytoplast could not be accomplish without the use of the cytoskeletal inhibitor cytochalasin B (CB) (McGrath and Solter, 1983). Treating mouse oocytes with CB prevented damage to the oocyte plasma membrane and allowed the insertion of a glass pipette through the zona pellucida without lysing the oocyte.

Enucleation of oocytes can be conducted at the metaphase II (MII) or telophase II (TII) of the oocyte cell cycle stage. When enucleation was conducted at MII stage,

PB-1 was used as a marker for the location of the MII plate and the efficiency of enucleating oocyte at this stage was reported to be less effective (Li *et al*., 2004). The problem with this approach is that in many cases the metaphase plate is not close to PB-1. This factor can result in a proportion of the oocytes containing residual DNA following enucleation. An alternative to the enucleation of MII oocytes is the enucleation of activated oocytes at telophase of the second meiotic division (telophase II: TII). Mechanical aspiration of the extruding second polar body (PB-2) and surrounding cytoplasm following activation is an effective and reliable enucleation method without the need for visualisation of the DNA by exposure to UV light. In addition, significantly less of the oocyte cytoplasm is removed when compared to enucleation of MII oocytes which may be beneficial to the developmental competence of the recipient cytoplast (Bordignon and Smith, 1998).

2.6.1.2 Transfer of donor karyoplast

Generally the two approaches that commonly used to introduce the donor karyoplast into the enucleated oocyte are: a) cell is transferred to the perivitelline space of the enucleated oocyte (Sub-Zonal Injection-SUZI) and subsequently electro-fused with direct current (DC) pulse; (b) donor cell is directly injected into the cytoplast of the enucleated oocyte (Whole Cell Intracytoplasmic Injection- WCICI).

The SUZI followed by electrofusion method was commonly used in cloning domestic animals and it has been successfully applied to produce viable cloned animals like sheep (Wilmut *et al*., 1997), cattle (Kato *et al*., 1998), goat (Baguisi *et al*., 1999), pigs (Polejaeva *et al*., 2000).

Electrofusion is the most widely used method in which the cell-oocyte couplets are placed in a largely nonionic, slightly hypotonic medium between two parallel electrodes and a high-voltage direct-current pulse delivered such that membrane breakdown is achieved (Zimmermann and Vienken, 1982). The pulse strength and duration must be determined experimentally, because conditions will vary slightly among different laboratories. The electrical pulse results in inversion of membrane phospholipids (breakdown), thus creating holes in the membrane; on healing, the membranes are fused. Therefore, too high a voltage for too long will result in lysis. However, if the voltage or duration is too low, poor fusion rates will be result.

The success of electric fusion requires precise alignment of the membranes to be fused perpendicular to the electric current, close contact between these membranes, and delicately timed species-specific duration and strength of the applied electric field (Zimmermann and Vienken, 1982). The electrical field strength (kV/cm), and the number and time interval of electric pulse (Ozil and Huneau, 2001) are believed to influence the developing capacity of reconstructed oocyte. Miyoshi *et al*. (2001) reported that a low fusion rate in cattle may occur when the orientation of the cell and enucleated oocyte to the electrode is not correct, there is little or no cell-to-cell contact, or both. Conversely, the efficiency of electrofusion seems to increase with the rise of the electric field strength and duration until these values reach a critical level. However, high levels of intracellular Ca^{2+} caused by the extension of pulse duration have a detrimental effect on the cell function or structures (Izant, 1983).

The WICIC technique can be carried out using conventional micropipette or with the Piezo-driven injection pipette. The WCICI approach involves the direct injection of donor cell into an enucleated oocyte, bypassing the fusion process. By using conventional micropipette, this approach is known to be less labour intensive, cost

effective while its efficiency is comparable with other approach (Lee *et al.*, 2003). On the other hand, the Piezo-driven WICIC approach is usually applied in cloning species such as mouse and horse in which the oocytes are fragile and their zona pellucidae are hard (Wakayama *et al*., 1998; Choi *et al*., 2002b). With this invention, the efficiency of the mouse cloning procedure has improved as the issue of hard zona pellucida was solved.

2.6.2 Activation

2.6.2.1 Mechanism of activation

The transition from mature oocyte to developing embryo requires a coordinated series of events, collectively known as egg activation. Egg activation includes changes to egg coverings to prevent polyspermy, release of oocyte meiotic arrest, generation of haploid female and male pronuclei, changes in maternal mRNAs and protein populations, and cytoskeletal rearrangements. In many animals, egg activation is triggered by sperm entry during fertilisation, which increases intracellular calcium within the oocyte (Swann and Ozil, 1994). These calcium oscillations thereby regulate the cascade of events including the cortical granule reaction (Miyazaki *et al*., 1990), zona pellucida reaction (Yanagimachi, 1994) and the escape from the metaphase-II arrest (Whitaker and Irvine, 1984). After a few hours, a series of events makes fertilisation complete, through the inactivation of maturation promoting factor (MPF) and of mitogen activated protein kinase that leads to resumption and completion of meiosis, DNA synthesis and pronuclei formation (Moos *et al*., 1996).

MPF was described as a complex of two subunits: a catalytic subunit, $p34^{cdc2}$ and a regulatory subunit, cyclin B. $p34^{cdc2}$ is a protein kinase regulated by changes in its

phosphorylation state and by its association with cyclin. Throughout the cell cycle, the level of $p34^{cdc2}$ remains constant but the level of cyclin varies. MPF peaks at metaphase in association with nuclear envelope breakdown, chromatin condensation, reorganisation of the cytoskeleton and the formation of the mitotic spindle (Doree and Galas, 1994; Moos *et al*., 1996). In order for the cell to exit MII arrest, inactivation of MPF is necessary and this involves cyclin proteolysis by the proteosome system (Glotzer *et al*., 1991).

In vertebrates, mature oocytes are arrested at metaphase-II of the meiotic division, with elevated MPF activity maintained by a cytostatic factor (CSF), which prevents the ubiquitin dependent degradation of cyclin B and thus, inactivation of MPF. Intracellular Ca^{2+} oscillations triggered by sperm down-regulate CSF activity and allow for the degradation of cyclin. Proteolytic degradation of cyclin B and subsequent MPF inactivation releases oocytes from metaphase arrest and allows the beginning or resumption of mitotic cycles (Lorca *et al*., 1993).

2.6.2.2 Artificial activation

However, in nuclear transfer, the absence of sperm-induced fertilisation steps leads to the requirement of artificial activation in order to trigger nuclear reprogramming and further embryonic development (Wells *et al*., 1999). Different artificial protocols have been developed to activate mammalian oocytes by simulating the biochemical and physiological events that normally occur during sperm-oocyte interaction as describe earlier.

Intracellular calcium rise can be induced in the oocytes without spermatozoa, using several chemical activating agents and electrical stimulation as a consequence,

oocyte is released from metaphase arrest but levels of MPF are not properly reduced and further development is impaired (Rinaudo *et al.*, 1997). In order to overcome this limitation, additional agents inhibiting protein synthesis or protein phosphorylation can be added and higher activation and *in vitro* development rates are observed in several species (Susko-Parrish *et al.*, 1994; Mitalipov *et al.*, 2001).

Calcium signaling can be stimulated using chemical agents such as Ca^{2+} ionophore, 7% ethanol, strontium chloride, phorbol ester and thimerosal (Nakada and Mizuno, 1998). Ionophore A23187 promotes the release of intracellular Ca^{2+} stores but also facilitates the influx of extracellular Ca^{2+} ions (Kline and Kline, 1992). Ionomycin is another potent Ca^{2+} ionophore currently used in NT protocols (Cibelli *et al.*, 1998; Wells *et al.*, 1999). It mobilises intracellular Ca^{2+} by depletion of Ca^{2+} stores. Exposure of matured oocytes to 7% ethanol for 5 to 7 min induces successful activation and pronuclear formation (Presicce and Yang, 1994) by promoting the formation of IP3 and the influx of extracellular Ca^{2+}. The substances mentioned above induce a single Ca^{2+} rise in the oocyte. However, the initial Ca^{2+} rise is normally followed by Ca^{2+} oscillations during fertilisation in mammals. Strontium chloride induces multiple Ca^{2+} transients probably by displacing bound Ca^{2+} in the oocyte (Whittingham and Siracusa, 1978) but also by inducing intracellular Ca^{2+} release (Kline and Kline, 1992). Strontium chloride has been successfully used to activate mouse oocytes after NT (Wakayama *et al.*, 1998).

Electrical stimulation is an alternative to chemical activation to induce Ca^{2+} influx through the formation of pores in the plasma. However, when this approach was used in a calcium and magnesium-free medium followed by incubation in 6-DMAP, the electroporation is known to induce the production of inositol 1, 4, 5-trisphosphate (IP_3) that leads to intracellular Ca^{2+} release. The combination of electroporation of IP_3 and 6-

DMAP has been used to activate parthenogenetic and NT rabbit embryos (Mitalipov *et al*., 1999; Liu *et al*., 2002a).

Inhibition of protein synthesis could be achieved using cycloheximide (CHX) while protein phosphorylation can be inhibited with 6- dimethylaminopurine (6-DMAP), an analogue to puromycin. Protein synthesis inhibitors (CHX) probably activate mammalian eggs by blocking the continuous synthesis of cyclin B that is required to stimulate the cell cycle protein kinase CDK1 (Moses and Kline, 1995). 6-DMAP appears to stimulate the tyrosine phosphorylation of $p34^{cdc2}$, consequently blocking the MPF activity (Jessus *et al*., 1991). However it is worth to note that detrimental effects of these non-specific, broad spectrum protein synthesis and/or kinase inhibitors are suspected during later embryonic development.

2.6.2.3 Production of parthenotes

Parthenotes can be efficiently obtained *in vitro* with a variety of mechanical, chemical, and electrical stimuli using oocytes of several species at different stages along oocyte meiosis resulting in parthenotes with different chromosome complements. According to the method of production, mammalian parthenotes can be either haploid or diploid. The developmental capacity of parthenotes is influenced by the resulting ploidy and genetic information. In some species, the development of both haploid and diploid parthenotes has been reported. These studies demonstrated that the haploid condition can impair cleavage at early stages (Escriba and Garcia-Ximenez, 2000; Kim *et al*., 1997; Lagutina *et al*., 2004).

Parthenogenetic activation can be performed in oocytes at the second metaphase resulting in the extrusion of the second polar body and leading to the formation of a

haploid parthenote. This method is rarely used since, in this case, the developmental competence is reduced compared to normal embryos and to diploid parthenotes (Henery and Kaufman, 1992). Diploid parthenotes are easily obtained: they can be derived from MII oocytes whose sister chromatids of the chromosome segregate without being extruded into the second polar body: thus the oocyte retains its diploid status, either homozygous or including cross-over associated heterozygosity in a single pronucleus. However, even the failure of diploid parthenotes to sustain a prolonged development highlights the need for biparental chromosome complement and, indirectly, the different and sometimes opposite developmental role of imprinted genes. Diploid parthenotes can be obtained in two main different ways. The most common one consists in combining the activation of MII oocytes with exposure to an inhibitor of the extrusion of the second polar body without affecting the formation and movement of pronuclei (Balakier and Tarkowski, 1967). Alternatively, a diploid parthenote can be generated by treating the oocyte with cytochalasin D during *in vitro* maturation before activation. This drug binds to the positive end of F-actin and blocks further addition of G-actin monomers preventing the extrusion of the first polar body. This protocol leads to the formation of tetraploid oocytes (Kubiak *et al*., 1991). The diploid status is then re-established at the end of oocyte maturation with the extrusion of the second polar body.

Using one or the other method has important consequences on the genetic make-up of the parthenotes. In fact, performing the oocyte activation before the inhibition of the second polar body extrusion determines the formation of highly homozygous parthenotes, since the diploid status of the parthenotes is obtained after the segregation of sister chromatids. In this case, the degree of heterozygosity depends only on the extent of crossing over taking place during the prophase of the first meiotic division which is very limited in most species (Rougier and Werb, 2001). On the contrary, extrusion of homologous chromosomes does not take place when the first polar body

extrusion is inhibited. In this case activation is induced in a tetraploid oocyte and the diploid status is reached again after the extrusion of the second polar body. As a consequence, the segregation of sister chromatids occurs only at this stage, therefore the parthenotes have the same proportion of chromosomes derived from the mother and the father of the donor as the oocyte and its donor. Parthenotes generated in this way, not only are genetically identical to each other but have the same heterozygosity of their mother (Kubiak *et al*., 1991).

2.6.3 *In Vitro* Culture

2.6.3.1 Culture medium

IVC of cloned embryos before transfer is one of the important steps in achieving pregnancy and delivery. Following NT and activation, bovine embryos are generally cultured *in vitro* for 6- 7 days prior to transfer. To date no *in vitro* embryo culture system rivals *in vivo* culture in terms of development efficiency and embryo quality when considering the outcome of any assisted reproduction approach. Although several system developed for the culture of *in vitro* fertilised embryos have been employed, it is unclear whether those are suitable for supporting preimplantation development of cloned embryos. The physiology of cloned embryos during early development has not been elucidated yet and if different requirement for exogenous substrate are found between IVF and cloned embryos, the composition of media employed should be further modified (Choi *et al*., 2002a). Suboptimal culture environment can affect the embryo morphology, embryonic growth, gene expression, postnatal growth and subsequently the phenotype (Kruip and denDaas, 1997; Summers and Biggers, 2003; Fleming *et al*., 2004; Thompson *et al*., 2007).

Caprine intraspSCNT embryos have been successfully cultured in different media such as TCM-199 (Baguisi *et al*., 1999), Charles Rosenkrans (CR1) (Guo *et al*., 2002; Lan *et al*., 2006; Chen *et al*., 2007), synthetic oviductal fluid (SOF) (Melican *et al*., 2005; Liu *et al*., 2011) and G1/G2 medium (Reggio *et al*., 2001) supplemented with serum or chemically defined compositions.

Serum generally contains beneficial substances for embryonic development and consequently foetal bovine serum (FBS) has been widely supplemented to embryo culture media. A higher developmental rate to the blastocyst stage was generally obtained from media supplemented with serum (Gardner, 1994; Pinyopummintr and Bavister, 1994; VanLangendonckt *et al*., 1997). However, it has been demonstrated that serum has a biphasic effect on embryonic development. The presence of serum in the medium may affect the speed of embryonic development, morphology, ultrastructure, metabolism and the gene expression profiles (Thompson *et al*., 2007). This is often associated with the large offspring syndrome (LOS) (Thompson *et al*., 1995; Sinclair *et al*., 1999). Therefore, almost all media used for embryo culture generally contain bovine serum albumin (BSA), instead of serum as a source of protein to improve embryonic development, blastocyst formation and the hatching rates (Thompson, 2000; George *et al*., 2008).

Glucose, pyruvate and lactate are important energy sources for mammalian embryos (Brinster *et al*., 1965). While glucose is necessary for the last part of IVC, pyruvate and lactate are important energy supplements for early stages of embryo development *in vitro* (Schini and Bavister, 1988). In general, embryos throughout pre-elongation development are reliant on oxidative phosphorylation via oxidation of pyruvate and amino acids. for the generation of ATP for embryo development (Javed and Wright, 1991; Rieger *et al*., 1992a,b; Gardner *et al*., 1993; Thompson *et al*., 1996,

2000). However, there is a switch to an increased contribution of glycolysis during compaction and blastulation (Gardner *et al*., 1993; Thompson *et al*., 1996, 2000). Failure to depress glycolysis during pre-compaction is one factor associated with the ‘‘8- to 16-cell’’ developmental block (Gardner *et al*., 1997; Thompson *et al*., 1992). Therefore, culture media should be designed to suppress glycolysis during pre-compaction development followed by removal of the suppression during post-compaction development. The former is often confused with complete removal of glucose from culture medium throughout pre-elongation development. However, this is unlikely to benefit the embryo, as glucose plays other roles including ribose and NADPH production through the pentose–phosphate pathway. In particular, ribose synthesis is important for the embryo, as this molecule is a precursor for DNA and RNA synthesis, which is essential for embryonic development. Such metabolic intermediates have been detected in sheep embryos following incubation with [U-^{14}C]- glucose, as well as incorporation into non-glycogen macromolecules (Thompson *et al*., 1995).

The requirements of early embryos as they progress through development and the temporal relationship between the reproductive tract fluid milieu and embryo development, has led to the concept that media components and physical conditions should be altered during culture to achieve improved development. This concept has subsequently been termed sequential media systems or two-step culture system (Gardner *et al*., 1997). Metabolic regulation is a relatively new concept, but has its origins before the beginning of this decade. Perhaps, the best known example of metabolic regulation is the use of ethylenediamine tetraacetic acid (EDTA), a non-selective chelator of divalent cations during embryo development. Several studies have shown that when *in vitro* glucose concentrations are higher than *in vivo*, early embryo development is retarded or even blocked (Seshagiri and Bavister, 1989; Chatot *et al*., 1989; Thompson *et al*., 1992).This has been likened to the ‘‘Crabtree-effect’’, whereby

oxidative phosphorylation is depressed due to an abnormally high glycolytic rate (Seshagiri and Bavister, 1989). Until recently, this was generally controlled by reducing the concentration of or even removing, glucose within the culture medium (e.g. Chatot *et al*., 1989). However, such conditions are unphysiological, as glucose is found in lumen fluids of the reproductive tract (Gardner *et al*., 1993). A previously unrelated observation is that EDTA addition during mouse *in vitro* embryo development overcame culture-induced development ‘‘2-cell’’ blocks (Mehta and Kiessling, 1990). It is believed that EDTA sequesters the toxic effects of contaminating heavy metal cations, most likely by inhibiting the production of reactive oxygen species, catalysed by ions such as $Fe^{2+}$and Cu^{2+} reviewed by Johnson and Nasr-Esfahini (1994). However, Lane and Gardner (1997) have demonstrated that, at a cellular level, EDTA depresses glycolytic rates within pre-compaction mouse embryos, a result also demonstrated for cattle embryos by Gardner *et al*. (1997).

2.6.3.2 Oxygen tension

Oxygen concentration in air (20%), which is generally employed for *in vitro* culture of embryos, is considerably higher than intraluminal oxygen tension in the reproductive tract of most mammals (Fischer *et al*., 1993). There are inconsistent reports regarding effects of low O_2 tension on *in vitro* development of preimplantation embryos. While some studies have reported beneficial effects (Fukui *et al*., 1991; Fujitani *et al*., 1997), others have reported either no effect (Betterbed and Wright, 1985), or even an adverse effect (Van der Westerlaken *et al*., 1992), of low oxygen concentrations on embryonic development. Improved embryo development in an atmosphere containing 5% oxygen compared with higher oxygen concentration has been achieved (Fukui *et al*., 1991; Fujitani *et al*., 1997; Lonergan *et al*., 1999). The

discrepancy in glycogen levels of blastocysts derived *in vitro* or obtained *in vivo* could be attributed to differences in the oxygen levels between the two systems (Khurana and Wales, 1989). Intrauterine oxygen concentration appears to be in the range of 1.5 to 7%. Poor development of embryos under atmospheric air (high oxygen concentration for example), may be attributed to the production of free oxygen radicals (Bavister, 1995). Even a short incubation (4 hours) of rabbit embryos at 20% O_2 led to alterations of gene expression indicating a severe stress response (Koerber *et al*., 1998). Other investigators have reported the necessity of using a high oxygen concentration for optimal embryonic development in the presence of co-cultures (Fukui *et al*., 1991; Nagao *et al*., 1994).

2.6.4 Cytoplast Source and Quality

In vivo sourced oocytes were used as recipient cytoplasts at the initial era of cloning. This was an expensive system, but with improvements to IVM procedures, much less expensive slaughterhouse derived oocytes could be substituted for *in vivo* oocytes (Keefer *et al*., 1993; Reggio *et al*., 2001). In mice *in vivo* derived oocytes are still used for SCNT. This fact is particularly intriguing when you consider the high developmental competency of *in vivo* sourced mouse zygotes as compared to the lower developmental competency of in vitro produced cattle or goat zygotes; yet, cattle and goat SCNT using *in vitro* produced oocytes results in much higher success rates on a perfused couplet basis than mice (Yang *et al*., 2007).

Oocytes and zygotes at different stages of development have been used as recipients for NT. Choice of metaphase II, telophase II or zygotic stage relates in part to coordination of cell cycles, but also to ease of enucleation, exposure to reprogramming factors and/or activated cytoplasm (Bordignon *et al*., 1998; Polejaeva *et al*., 2006).

2.6.5 Donor Cell

2.6.5.1 Selection of donor cell type and duration of cell culture

Various cell types, such as embryonic cells (Campbell *et al*., 1993, 1996; Cheong *et al*., 1993), fibroblasts (Kato *et al*., 1998), mammary gland cells (Wilmut *et al*., 1997), cumulus granulosa cells (Wakayama *et al*., 1998), oviduct cells (Kato *et al*., 2000), leukocytes (Galli *et al*., 1999), mural granulosa cells (Wells *et al*., 1999), germ cells (Bordignon *et al*., 2003), embryonic stem cells (Eggan *et al*., 2001) and liver cells (Brem and Kuhholzer, 2002) have been used as donor cells for production of cloned animals. However, it is still unclear which type is the most efficient for nuclear transfer into oocytes (Kato *et al*., 2000).

Initial study on the production of cloned animal used adult somatic cells derived from female reproductive system such as mammary gland (Wilmut *et al*., 1997), oviduct (Kato *et al*., 1998), cumulus and mural granulosa cells (Wakayama *et al*., 1998; Wells *et al*., 1999; Kato *et al*., 2000). However, the limitation of using these cell type is only female cloned could be generated if SCNT was performed using these cell type.

Foetal cells are believed to have less genetic damage and more proliferative ability (as measured by cell doublings) than adult somatic cells, they have been the cell type of choice as nuclear donors (Hill *et al*., 2000). However, this presents a limitation in that the individual the donor cells are derived from (a foetus) has not had the chance to demonstrate its genetic merit (an adult) prior to somatic cell nuclear transfer.

Genetic damage may occur during the *in vitro* culture of donor cells prior to NT. Culturing somatic cells especially for prolonged periods is known to alter ploidy, genomic stability and post-translation histone modifications, factors which are known to reduce cloning efficiency (Jang *et al*., 2004). Therefore, fresh or short-term cultured

(<10 subpassages) donor cells have been the cell type of choice for the production of cloned embryos. In contrast, one study reported higher developmental rates to the blastocyst stage for embryos reconstituted with adult somatic cells that had been sub-passaged 10 to 15 times compared with that of embryos reconstituted from cells with a lower number of sub-passages (Kubota *et al*., 2000). In addition, cloned calves were obtained from embryos reconstituted with high sub-passaged cells, with all cloned foetuses derived from low sub- passaged donor cells aborting during pregnancy (Kubota *et al*., 2000). Results within and among various research laboratories are often conflicting due to procedural effect, oocyte variability, inherent differences among donor cell lines, age of donor animals or to effects of *in vitro* culture conditions..

2.6.5.2 Stages of donor cell cycle

The stage of the donor cell cycle is a major factor in the success of nuclear transfer in mammals (Wilmut *et al*., 1997; Campbell *et al*., 1996). Quiescent donor cells arrested in the G1 or G0 stage of the cell cycle have been used to produce mice (Wakayama *et al*., 1998), pigs (Onishi *et al*., 2000; Polejaeva *et al*., 2000), sheep (Wells *et al*., 1997), cattle (Wells *et al*., 1999) and goat (Baguisi *et al*., 1999). Methods of arresting cells in this phase of the cycle have been explored using reversible cycle inhibitors (Alessi *et al*., 1998), non-pharmaceutical treatments such as mitotic shake-off to select recently divided cells in G1 or contact inhibition to select cells in G0/G1 (Boquest *et al*., 1999; Kasinathan *et al*., 2001), serum starvation (Kues *et al*., 2000) and cell confluency as a donor cell treatment prior to nuclear transfer. The specific method used to arrest donor cells can markedly affect foetal survival to term and neonatal survival (Gibbons *et al*., 2002). However, proliferating cells have also been successfully used for NT (Cibelli *et al*., 1998), although the exact stage of the donor cell cycle was never verified. Thus far,

only statistical probabilities on cell stage percentages in G0/G1, G2, M and S-phases have been provided as evidence of the cell cycle stage (Boquest *et al*., 1999).

Roscovitine or nocodazol is a cyclin dependent kinase (CDK) 2 inhibitor that effectively arrests fibroblasts in the G0/G1 quiescent phase of the cell cycle (Alessi *et al*., 1998) and can be used to maintain bovine oocytes at the germinal vesicle stage of maturation by inhibiting meiosis promoting factor, a member of the CDK family (Mermillod *et al*., 2000). Following roscovitine removal, cells arrested in G0/G1 resumed cycling and entered the S phase as expected (Alessi *et al*., 1998), and oocytes arrested at the germinal vesicle stage progressed to metaphase II (Mermillod *et al*., 2000), indicating that the effects of roscovitine were fully reversible. In the SCNT research, the use of cell cycle synchronised donor cell probably impacts the capacity of the nucleus to be reprogrammed due to the reduced transcriptional efficiency in the quiescent cell; however, this hypothesis has not been tested.

2.6.6 Epigenetics

Epigenetics refers to stable and heritable changes in gene expression beyond the scope of conventional genetics. In other words, gene expression in a cell is not controlled exclusively by the DNA sequence, but also by these stable "epigenetic" influences to specific genes (Jaenisch and Bird, 2003). In addition to their importance in differentiation of tissue types during development, epigenetic alterations can also arise randomly or as a result of environmental influence. The genome adapts to developmental or environmental cues either by post-synthetic modification to DNA or by modification of proteins associated with DNA. It is believed that epigenetic modifications have arisen and evolved as a genome defense against viruses and other parasitic sequences (Matzke *et al*., 1999). Cellular differentiation occurs as a

consequence of epigenetic modifications imposed upon the genome. These epigenetic modifications direct the expression patterns of cell-type-specific genes; therefore SCNT necessitates the reprogramming of the donor cell carrying cell-type specific epigenetic modifications in order for every cell type to be derived from a once-differentiated donor cell. It is widely believed that incomplete or improper epigenetic reprogramming following SCNT results in the low efficiency as well as the phenotypic problems observed in clones.

A variety of epigenetic modifications to DNA and its associated proteins have been characterised. These modifications can serve either to silence expression or to enhance transcription of specific genes. Predominant epigenetic modifications include DNA methylation, and modifications to histones including methylation, acetylation, ribosylation, phosphorylation, and ubiquitination (Glotzer *et al.*, 1991).

2.6.6.1 Epigenetic reprogramming following nuclear transfer

Epigenetic reprogramming is essential in order for SCNT be successful. During nuclear reprogramming epigenetic marks are erased from the donor nucleus genome, resulting in an erasure of tissue-specific gene expression patterns effectively resetting the cell to a totipotent state (Santos and Dean, 2004). Studies evaluating the epigenetic status of embryos following SCNT have demonstrated deficiencies in epigenetic reprogramming frequently occur. These deficiencies are made manifest in several ways including changes in histone modifications, DNA methylation patterns, and gene expression.

Several studies have evaluated differences in epigenetic modifications following SCNT. Hypermethylation of lysine 9 on histone H3 (H3-K9) as well as DNA hypermethylation was reported in the majority of bovine preimplantation SCNT

embryos in one study (Santos *et al*., 2003). A number of studies evaluating the reprogramming dynamics of epigenetic modifications *in vitro* following NT have also been reported. Evaluation of DNA methylation patterns in developing NT embryos indicates demethylation and remethylation events are not always faithfully recapitulated in the mouse (Chung *et al*., 2003; Shi and Haaf, 2002) and the cow (Bordignon *et al*., 2001; Dean *et al*., 2001). It is also clear that histone acetylation is sometimes aberrant in bovine SCNT embryos (Enright *et al*., 2003). This incomplete epigenetic reprogramming is the predominant explanation for the frequent aberrant gene expression in NT embryos and the subsequent failures in development (Santos *et al*., 2003).

Immunofluorescent staining of bovine NT embryos with an antibody directed against 5-methyl-cytosine by Dean *et al*. (2001) demonstrated the occurrence of active demethylation of the donor chromatin shortly after fusion similar to the active demethylation of sperm chromatin observed following fertilization (Dean *et al*., 2001). However, de novo methylation occurred starting in 4-cell NT embryos as compared to normal bovine embryos, which exhibit de novo methylation at the 8- to 16-cell stage (Dean *et al*., 2001). The enzymes responsible for DNA demethylation may follow a pattern of activity similar to maturation promoting factor (MPF) with high activity prior to activation and a diminishing of activity following activation. Further research will be required to determine those dynamics, but based on one study there appears to be a critical window of time in which active demethylation can occur following fusion (Dean *et al*., 2001). The idea of this critical window between fusion and activation is supported by the work of Bourc'his *et al*. (2001) in which active demethylation was not observed when activation was performed at the time of fusion (Bourc'his *et al*., 2001).

Other studies have focused on epigenetic reprogramming of specific genes following SCNT. Evaluation of methylation patterns of imprinted genes following SCNT indicates methylation errors at imprinted loci are common (Mann *et al*., 2003). In addition, errors in X-inactivation (Eggan *et al*., 2000) and failures to activate important pluripotency genes have been observed in SCNT embryos (Boiani *et al*., 2002). Critical errors in the fundamental epigenetic state of chromatin during early development following SCNT are likely the foundation for the numerous other deficiencies observed in clones (Fulka and Fulka, 2007).

The use of chromatin remodeling agents such as Trichostatin A (TSA), scriptaid and m-Carboxycinnamic Acid Bishydroxamide (CBHA) was reported to be able to improve epigenetic reprogramming during nuclear transfer (NT) (Kishigami *et al*., 2006; Nguyen *et al*., 2009; Dai *et al*., 2010). These chromatin remodeling agents function as histone-deacetylase inhibitor (HDACi), which enhances the pool of acetylated histones (Yoshida *et al*., 1990; Dai *et al*., 2010) and DNA demethylation (Hattori *et al*., 2004) which were important in modifying the epigenetic configuration.

2.7 EMBRYO TRANSFER

The first embryo transfer (ET) in goat was reported by Warwick *et al*. (1934). In early sixties, fundamental and physiological studies related to ET in small ruminant were performed by several famous scientists (Thibier and Guerin, 2000). Three methods of ET namely, laparoscopic ET, surgical ET and transcervical ET have been reported in small ruminants (Stefani *et al*., 1990; Flores-Foxworth *et al*., 1992) of which laparoscopic ET is more successful (Abdullah *et al*., 1995; Ishwar and Memon, 1996). The success of ET depends on several factors including management of recipient does,

oestrus synchronisation in recipients and the technical skill during transfer (Ishwar and Memon, 1996).

Developmental anomalies produced by SCNT after embryo transfer include low pregnancy rates, an unacceptably high level of losses during early and late pregnancy, stillbirths, early postnatal deaths, short life-span, obesity and malformations. The term most frequently used to describe some of these developmental anomalies is “large offspring syndrome” (LOS). Essentially, LOS refers to increased birth weight. The occurrence of LOS seems to be species-specific: it is quite frequent in cattle, sheep and mice, and some elements have also been described in humans after assisted reproductive procedures. However, almost no malformations were detected in pigs and goats (Betthauser *et al*., 2000; Reggio *et al*., 2001; Behboodi *et al*., 2005), although these results require confirmation.

Chapter 3

3.0 MATERIALS AND METHODS

Chapter 3

3.0 MATERIALS AND METHODS

3.1 INTRODUCTION

The main objectives of this study were to produce cloned caprine embryos and kids via intraspecies- and interspecies somatic cell nuclear transfer approaches (intraspSCNT and interspSCNT, respectively) as well as to develop nuclear transfer protocol using skin fibroblast cell as donor karyoplast for our laboratory. In experiments pertaining to caprine intraspSCNT, oocytes as recipient cytoplasts were obtained from two sources, namely abattoir derived ovaries and laparoscopic ovum pick-up (LOPU) from superstimulated does. As for caprine interspSCNT, oocytes were obtained from abattoir derived bovine ovaries. The ovary sample collections from abattoir for both species were carried out once or twice weekly, depending on the availability of females to be slaughtered. Oocyte retrieval via LOPU surgery was conducted once a week on either one or two superstimulated does in each LOPU session. All the media and reagent preparations, processing of abattoir and LOPU oocytes retrieval, nuclear transfer and embryo transfer surgeries were carried out at the Nuclear Transfer and Reprogramming (NaTuRe) Laboratory, Institute of Research Management and Monitoring (IPPP), University of Malaya. The duration of the entire caprine intraspSCNT and interspSCNT research was carried out in this laboratory between November 2008 and April 2011.

3.2 MATERIALS

The following materials were used in this study: such as livestock ovarian and ear tissue samples, various equipment, chemicals, reagents and media as well as labwares and disposables. Each component mentioned above will be described briefly in the subsequent sections:

3.2.1 Livestock Ovarian and Ear Tissue Samples

Caprine and bovine oocytes were used as the recipient cytoplasts for the production of cloned embryos and parthenotes. While caprine, bovine and gaur ear skin tissues were biopsied to develop skin fibroblast cell lines that served as the donor karyoplasts for the SCNT research. The caprine oocytes were retrieved either by LOPU on superstimulated does or slicing of ovaries collected from abattoir source. On the other hand, the source of bovine oocytes was solely retrieved from abattoir-derived ovaries.

3.2.1.1 Experimental does

A total of 45 does were used in the present study of which 33 does were assigned for LOPU oocyte retrieval donors and 12 does for embryo transfer recipients. All the does selected for this study were represented by various breeds of caprine such as Boer crossbred, Jamnapari and mixed breeds (crosses among breeds namely Jermasia, Katjang, Boer and Jamnapari) that aged between 6 and 48 months old at the time of experiments. All the does used in this entire study for oocyte retrieval were kept and well maintained in the ISB Mini Livestock Farm, University of Malaya in accordance with the animal welfare guidelines. All the does were fed with a sufficient diet

consisting of Napier grass and pellet feed (goat/sheep pellet, KMM Berhad, Malaysia) and given water *ad libitum*.

3.2.1.2 Abattoir-derived ovaries

A total of 96 caprine ovaries and 132 bovine ovaries were obtained from the abattoir for oocyte retrieval. Both caprine and bovine ovaries used in this study, except for Experiment 1 and 3 (preliminary study for goat SCNT), were collected from Abattoir Complex, Department of Veterinary Services, Shah Alam, Selangor and Senawang, Negeri Sembilan, Malaysia, whenever the ovary samples were available. Bovine ovaries used in Experiment 4 were collected from Abattoir Complex Praputtabaht, Saraburi, Thailand as this preliminary study was conducted in Embryo Technology and Stem Cell Research Centre (ESRC), Suranaree University of Technology, Nakhon Ratchasima, Thailand (between Oct 2009 and Dec 2009). All the ovaries were transported to the laboratory in thermos flask (37°C) within duration of 1 to 3 hours after slaughter. The ovary transporting medium (Table 3.2) consisted of normal saline (0.9%; Sigma-Aldrich Co., USA) supplemented with penicillin and streptomycin (Sigma-Aldrich Co., USA). The ovaries were collected from does and cows that their breed, origin, health condition, phases of oestrus cycle and pregnancy were unknown as the records were not available.

3.2.1.3 Source of ear skin fibroblast cell

Caprine ear skin fibroblast cell lines used in this study were cultured from ear skin tissue biopsied from caprine of Jermasia and Boer breeds reared in ISB Mini Livestock Farm, University of Malaya and Rumpun Asia Sdn. Bhd, Ulu Yam, Selangor. On the

other hand, the bovine and gaur skin fibroblast cells were obtained from the available cryopreserved cell line bank developed in ESRC, Thailand. Both male and female caprine, bovine and gaur skin fibroblast cell lines were used as the donor karyoplast for the production of cloned caprine, bovine and gaur embryos, respectively. As for caprine, criteria for karyoplast selection was based on special characteristics such as body conformation and health of the donors for both sexes.

3.2.2 Equipment

The list of equipment with details such as model number, manufacturer and supplier's name used in this present study are presented in Appendix Table 1.1. The major equipment used comprised of autoclave, centrifuge, CO_2 incubator, electrofusion machine, fluorescent microscope, flushing and aspiration system, freezer (-80ºC) inverted microscope with micromanipulators, laminar air flow work station, liquid nitrogen tank, microforge, micropipette grinder, micropipette puller, osmometer, oven, pH meter, stage warmer, stereomicroscope, surgical set, ultrapure water purification system and water bath.

3.2.2.1 Inverted microscope and micromanipulation system features

The core equipment for SCNT manipulation requires an inverted microscope that is attached with micromanipulation system. An Olympus inverted microscope (IX71; Olympus Optical Co., Ltd, Japan) that fitted with objective lenses (4x, 10x, 20x and 40x) and Hoffman modulation contrast system (Normaski system) was utilised. This inverted microscope was fitted with additional features such as the transparent stage warmer (37°C), camera and a connection to the personal computer.

The hydraulic micromanipulation system used in conjunction with this microscope comprised of the following components:

1) The first component consists of a set of left and right arms attached to the microscope stage via microscope mounting adapter (ON-IX99-2). The left and right arms with the universal joints (UT-21) on both side served to clench the stainless holding pipette and enucleation or injection pipette holder, respectively.
2) The movements of the left and right arms are controlled by a pair of drop handle joysticks and a pair of motorised positioners. The drop handle joysticks through an oil hydraulic mechanism controlled the three-dimensional fine (X-Y-Z axes) positioning of the microtools. While the three-dimensional coarse movements were performed electrically with the motorised positioners.
3) The holding and injection units were controlled with air. Both the units consist of a stainless microtool holder connected to a completely airtight syringe (3 ml) through the polystyrene tubing. While the enucleation pipette (squeezing needle) unit is attached to an electrode holder (H-7) without the need of air control.

3.2.2.2 Electrofusion machine

The donor karyoplast was introduced into the enucleated oocyte via electrofusion method. The needle fusion approach was adapted whereby the cell fusion system (SUT F-1; Suranaree University of Technology, Thailand) connected to a pair of platinum electrodes was used. The platinum electrodes were attached to the left and right arms of the micromanipulator. Both electrodes were aligned at the bottom of the petri dish containing fusion medium. Movement of the electrodes was controlled by the left and right joystick.

3.2.3 Chemicals, Reagents and Media

All the solutions and media used in this research were prepared using analytical grade laboratory chemicals and reagents. Most of the chemicals, reagents and media used were purchased from Sigma-Aldrich Co., USA, unless otherwise stated. A detailed list of the chemicals, reagents and media with catalogue number, manufacturer's and supplier's company name is presented in Appendix Table 1.2.

3.2.4 Labwares and Disposables

In general, the main disposables used for oocyte retrieval and SCNT studies involved graduated test tubes, CE marked dishes for IVF (35 mm diameter), polystyrene with surface treated for cell culture dishes (35 and 60 mm diameter) and polystyrene with poly-D-lysine surface coated culture flasks (75 and 175 cm^2 culture area). A detailed list of labwares and disposables with manufacturer's name used in the study is tabulated in Appendix Table 1.3

3.3 METHODOLOGY

Generally, the description of the research methodology section involved 4 main subsections, namely: 1) The standard maintenance of research laboratory, 2) Preparation of stocks and media, 3) Preparation of microtools and 4) Experimental procedure.

3.3.1 Standard Maintenance of Research Laboratory

The general maintenance of a laboratory specifically related to living cell culture involves strict observation of cleanliness practices. In order to obtain favourable

outcome in any IVP endeavour, the organisation of the laboratory structure is ensured to be free from distraction and the risk of accident as well as the contamination possibilities in the laboratory are kept to the minimal level. Throughout the research, the following elements of aseptic practices and maintenance precaution were observed.

3.3.1.1 Personal hygiene & safety

The general practices of personal hygiene observed in the laboratory include, wearing of clean lab coat, appropriate face mask, hair cap and gloves (if necessary). Besides that, the practice of washing hand with disinfectant before and after any experiment is a must to reduce loosely adherent microorganisms on the skin that would otherwise be likely to be blown onto the culture.

3.3.1.2 Work surface cleanliness and sterilisation

Prior commencing any experiment, the surface of the working bench, laminar flow hood, microscopes stage and surface of any equipment were swabbed liberally with ethanol (70%) for sterilisation purpose. The residual traces of ethanol (70%) were allowed to evaporate for at least 10 to 15 minutes before commencing any of the experimental work. Any spillages that occurred during the experiment were wiped immediately with tissue paper and the surface was swabbed with ethanol (70%). Once the work completed, all the apparatus on the working surface were arranged back to its respective storage place and ultimately the entire working surfaces were swabbed again with ethanol (70%). In order to maintain sterility of the laminar flow hood, the ultraviolet (UV) light in the laminar flow hood was switched on overnight.

3.3.1.3 Labwares cleanliness and sterilisation

Cleaning of all glassware and non-disposable items used in this study such as Scott bottles, beakers, volumetric flasks, measuring cylinders, magnetic stirrer, conical tubes and etc. was done scrupulously. All the used labwares were rinsed with tap water to remove traces of chemical before soaking in diluted detergent (7X®-PF) overnight. The labwares were brushed and rinsed thoroughly in four complete changes of tap water followed by three changes of deionized water or reverse osmosis (RO) water. After the washing process was completed, all the labwares were dried upside down in the oven (60°C). Dried glasswares that did not have an attached cap such as beakers, volumetric flasks and measuring cylinders were covered with aluminium foil. On the other hand, screw-cap glasswares were loosely capped (half to one turn so that they stayed on) and covered with aluminium foil. As for other clean-dried, non-disposable item such as screw-capped conical tube, the cap was loosely screwed on the top before packed in autoclavable bag. A piece of autoclave tape labelled with the researcher's ID and date was placed on all the cleaned labwares before sterilising in the autoclave machine for 20 to 25 minutes at 121°C, 15 psi. After sterilisation, all the labwares were dried in the oven before placing it in the appropriate storage cabinets which were securely covered. In order to prevent vacuum forming in the glass bottle after removing from the oven, the cap of the bottle was untightened until it was completely cooled to room temperature (25°C).

On the other hand, sterilisation for the non-autoclavable labwares such as polystyrene culture dishes, microtools for the SCNT procedure, oocyte collection tubes and mouth piece were conducted using the application of UV light for at least 2 hours inside the laminar flow hood.

3.3.1.4 Maintenance of embryo culture environment (CO_2 incubator)

The IVP of embryos in this laboratory used a chamber with CO_2 (5%) in humidified air to maintain the correct physiological pH (pH 7.3 to 7.4) and a temperature of 38.5°C. The accurate calibration of incubator was ensured and monitoring of the temperature with an external temperature check device was conducted weekly.

Besides that, the cleanliness of the incubator was done monthly according to the regime that involved wiping the inner surface of the incubators and door with ethanol (70%). The residual traces of ethanol were ensured to evaporate completely before using the incubator. The tray and racks of the incubator were washed with mild detergent (7X®-PF) followed by a final rinse with sterile reverse osmosis (RO) water. Prior placing the tray and racks back into the incubator, it is important to ensure that the tray and all racks were completely dried and sterilised using UV light for 30 minutes. The tray was then filled up with sterile RO water to generate a humidified environment in the incubator. In order to maintain a stabilised culture environment, repeated opening and closing of the incubator's door was kept to the minimum.

3.3.2 Preparation of Stock Solutions and Media

Preparation of any culture media requires accurate measurements of each chemical component. In order to achieve this, preparation of stock solutions in a concentrated form is required. The practice of stock solutions preparation also enables experimenter to save time and conserves materials during the subsequent weekly culture media preparation process.

Water forms the most basic and common component in all stock solution and medium preparation, thus it is vital to use a reliable source of treated ultrapure water.

All the media used in this study were prepared from scratch, using embryo or tissue culture-tested chemicals and water. The treated water was processed via Milli-Q®- RO Pulse pure water system that combines reverse osmosis and electrodeionisation (EDI) components to yield high quality surface water. Pre-filtered water first passed through reverse osmosis which removed 95-99% of a broad spectrum of organics, ions, bacteria, pyrogens and particles. Water processed through reverse osmosis was directed to an EDI module which used a combination of ion-exchange (IE) membranes, IE resin and direct current to remove ions from water. The Milli-Q®- RO Pluse product water was sent to a temporary storage reservoir in which water was recirculated for 2 hours/day through a 185 nm UV oxidation system which oxidised organics and destroyed bacteria in the stored water to reduce any inherent contamination. The reverse osmosis performance was monitored daily by recording the percentages of ionic rejection, feed and product conductivity as well as temperature. The acceptable rejection rates ranged from 95-99%. While EDI purification must be able to provide 10-15 MΩ-cm water with < 30 parts per billion (ppb) total organic carbon (TOC).

The Milli-Q®- RO Pluse -processed water in the storage reservoir was supplied to a Milli-Q® PF Plus ultrapure water system. Within the Milli-Q PF system, the water was further processed through purification pack which contained nuclear-grade, high purity, IE resin and a special organic scavenging resin. This process produced 18.2 MΩ-cm water with organic levels < 8 ppb TOC. The ultrapure water was further purified through a 5000 Da hollow-fibre ultrafilter. All proteins, particles, organics and endotoxins (> 5000 Da) were removed as the water was processed from the exterior into the interior lumen of the fibres. The final polished water was then passed through a membrane filter (0.22 µm) to scavenge any trace particles from the water and also to prevent reverse bacterial contamination from incoming air from the environment.

The preparation of fundamental stock solutions and media were conducted in the laminar air flow work station. All the chemicals in powder form were weighed using digital analytical balance while chemical in liquid form were measured using disposable plastic pipette, micropipette or measuring cylinder. In order to facilitate the dissolving and mixing of chemical components in the medium preparation (except medium that contain serum and PVP), a magnetic stirrer with moderate speed was used.

The fresh oocyte or embryo culture media prepared weekly or biweekly were subjected to pH and osmolarity measurements. The pH and osmolarity of the media were adjusted to 7.2 to 7.4 and 280 to 300 mOsm/kg, respectively prior use. The stock solutions and media were filtered once again using syringe filter (0.22 µm pore size) for sterility assurance prior aliquot. All the bottles or tubes that were used to keep the stock solution and media were labelled (Name and concentration of the media, ID of the personnel and date of preparation) and sealed with parafilm prior storing in the refrigerator (3-5°C) or freezer (-20°C) appropriately.

3.3.2.1 Preparation of normal saline solution

Normal saline solution is 0.9% (w/v) sodium chloride in water and it is also known as a physiological solution. The isotonic property of normal saline is suitable to prevent dehydration of live tissue, thus it was used for washing and collection of any tissue specimen in this study. Normal saline was prepared in a Scott bottle (1 litre) by dissolving sodium chloride (9 g) in Milli-Q water (1 litre). The normal saline solution was autoclaved at 115°C, 10 minutes and kept for 3 months in the refrigerator (3-5°C) for future use.

3.3.2.2 Preparation of heparinised saline solution

In this study, heparinised saline solution is required during LOPU and embryo transfer surgeries. Generally, animals that undergo abdominal surgeries such as those on the uterus, and ovaries are prone to have adhesions as the body heals inside. Adhesions are bands of fibrous scar tissue that form on abdominal organs, causing the organs to stick to one another or to the wall of the abdomen. Thus as a preventive measure to the occurrence of adhesion after the LOPU and embryo transfer surgeries, heparinised saline solution was filled into the abdomen or pelvic area of the does before suturing the incised wound. The heparinised saline solution will linger in the abdominal cavity for a few days before it is absorbed, which prevents any major adhering.

The heparinised saline solution was prepared by dissolving heparin (0.05 g) in the autoclaved normal saline (1000 ml) as depicted in Table 3.1. The solution was mixed well using magnetic stirrer with moderate speed. This solution can be kept for 1 month in the refrigerator (3-5°C) for future use.

Table 3.1: Composition of heparinised saline solution

Chemical component (catalogue number)	Final concentration	Quantity/1000 ml
Autoclaved normal saline	1 x	1000 ml
Heparin (H0777)	7 USP/ml	0.05 g

(Storage temperature: 3-5°C; shelf life: 1 month)
Note: 1 mg of heparin contains 140 USP units.

3.3.2.3 Preparation of ovary collection medium

Ovary collection medium (OCM) was prepared for the purpose of washing and collecting ovaries from abattoir. The OCM consisted of normal saline (0.9% NaCl) solution supplemented with penicillin G sodium salt and streptomycin sulphate salt that serve as antibiotic agents. In order to prepare OCM with the final volume of 1000 ml as depicted in Table 3.2, penicillin G sodium salt (60 µg/ml) and streptomycin sulphate salt (50 µg/ml) were weighed and dissolved in normal saline solution (1000 ml). Prior ovary collection, the required amount of OCM in use was aliquot and warmed in the water bath at 37-38°C. Extra OCM can be stored in the refrigerator (3-5°C) with the shelf life of 1 month for future use.

Table 3.2: Composition of ovary collection medium

Chemical component (catalogue number)	Final concentration	Quantity/1000 ml
Autoclaved normal saline	1 x	1000 ml
Penicillin G sodium salt (P3032)	0.17 mM	0.06 g
Streptomycin sulphate salt (S1277)	0.07 mM	0.05 g

(Storage temperature: 3-5°C; shelf life: 1 month)

3.3.2.4 Preparation of phosphate-buffered saline solution without calcium chloride and magnesium chloride, PBS(-)

Generally, PBS(-) solution was prepared for dual purpose. It was used as diluent for the preparation of certain chemical stock solution as it is a form of balanced salt solution.

Besides that, it was used in tissue culture work for washing and suspending cells. PBS(-) was prepared according to the list of chemical composition depicted in Table 3.3. All the chemicals in powdered form were weighed and dissolved one after another, according to sequence in Milli-Q water dispensed in a Scott bottle (500 ml). A magnetic stirrer at moderate speed was used to facilitate the dissolving and mixing of the chemical component in the solution. The well mixed PBS(-) solution was filter-sterilised using syringe filter (0.22 µm pore size) before use. The solution can be kept in the refrigerator (3-5°C) with the shelf life of not more than 3 months.

Table 3.3: Composition of PBS(-)

Chemical components (catalogue number)	Final concentration (mM)	Quantity/500 ml
NaCl (S5886)	171	5.00 g
KCl (P5405)	3.35	0.125 g
Na_2HPO_4 (S5136)	10.1	0.72 g
KH_2PO_4 (S5655)	1.84	0.125 g
Milli-Q water	-	500 ml

(Storage temperature: 3-5°C; shelf life: 3 months)

3.3.2.5 Preparation of penicillin G sodium salt and streptomycin sulphate salt stock solution [100x]

The penicillin G sodium salt and streptomycin sulphate salt (PS) stock solution with the concentration of 100x was prepared according to the composition presented in Table 3.4. The stock solution was filter-sterilised (0.22 µm pore size) before aliquot in sterile

microcentrifuge tubes (200 µl). PS stock solution was then stored in the freezer (-20°C) with the maximum shelf life of 6 months.

Table 3.4: Composition of PS stock [100x] solution

Chemical component (catalogue number)	Final concentration	Quantity/10 ml
PBS(-) solution	1 x	10 ml
Penicillin G sodium salt (P3032)	168.4 mM	0.6 g
Streptomycin sulphate salt (S1277)	137.2 mM	1.0 g

(Storage temperature: -20°C; shelf life: 6 months)

3.3.2.6 Preparation of oocyte retrieval medium

In this study, Dulbecco's phosphate buffered saline (DPBS) supplemented with polyvinylpyrrolidone (PVP), average molecular weight of 360,000 (PVP-360) was used as oocyte retrieval (OR) medium. DPBS is a balanced salt solution supplemented with the buffer's phosphate group to maintain media in the physiological pH range (7.2-7.4) and at the same time maintaining cell tonicity. In order to prevent oocyte from sticking to glass or tissue culture ware during oocyte manipulation, PVP-360 was supplemented as a source of surfactant.

In OR via LOPU procedure, this medium was used as flushing medium whereby microvolumes of this medium was flushed into the ovarian follicles on the surface of the ovary via an aspiration system (a vacuum pump) and subsequently, the follicular fluids containing oocyte from the follicles were aspirated into a sterile round-bottom test tube (15 ml) which was pre-warmed (37°C) by a test tube heating system. Each

collection tube was used to collect the aspirated fluids of approximately 3 ml before passing the tube to the embryologist for oocyte searching under the dissecting microscope. Similar medium was also used in OR from abattoir derived ovaries for rinsing and slicing ovary as well as for oocyte collection.

The DPBS stock solution was prepared according to the composition depicted in Table 3.5. The DPBS was prepared in a one litre bottle by dissolving 10 tablets of DPBS (Dulbecco A, BR0014G; Oxoid Ltd., England) in one litre of Milli-Q water (one tablet in each 100 ml of Milli-Q water). A magnetic stirrer at moderate speed was used to facilitate the dissolving and mixing of the chemical component in the solution. Subsequently, PS [100x] stock solution (1 ml) was added and the final solution was filter-sterilised (0.22 µm pore size) before kept in the refrigerator (3-5°C) with the shelf life of 1 month.

Table 3.5: Composition of DPBS stock solution

Chemical component (catalogue number)	Final concentration	Quantity/1000 ml
PBS tablets (Dulbecco A, BR0014G)	1x	10 tablets
PS stock [100x]	1x	1.00 ml
Milli-Q water	-	1000 ml

(Storage temperature: 3-5°C; shelf life: 1 month)

The working solution for oocyte retrieval was prepared by adding PVP-360 powder (0.5 g) into DPBS stock solution (500 ml) dispended in a Scott bottle (Table 3.6). The PVP-360 was left to dissolve on its own in the DPBS stock solution without

any stirring motion to avoid foam formation. Thus, the preparation of oocyte retrieval medium was made preferably 12 hours prior use to ensure the PVP-360 was dissolved completely. For oocyte retrieval medium or flushing medium used in LOPU procedure, heparin (0.018 g) was supplemented.

The oocyte retrieval solution was then filter-sterilised (0.22 µm pore size) before using and the solution can be kept in the refrigerator (3-5°C) for future use, not more than 2 weeks.

Table 3.6: Composition of oocyte retrieval medium (working solution)

Chemical component (catalogue number)	Final concentration	Quantity/500 ml
DPBS stock solution	1 x	500 ml
PVP-360 (Sigma, PVP-360)	0.1%	0.50 g
*Heparin (H0777)	5 USP/ml	0.018 g

(Storage temperature: 3-5°C; shelf life: 2 weeks)
Note: * Heparin was only supplemented for oocyte retrieval medium used in LOPU.

3.3.2.7 Heat-inactivation of foetal bovine serum

Serum is known to contain growth factors, which promote cell proliferation, adhesion factors and antitrypsin activity, which promote cell attachment. It is also a source of minerals, lipids and hormones, many of which may be bound to protein. Besides that, serum could also regulates cell membrane permeability and serves as a carrier for lipids, enzyme, micronutrients and trace elements into the cell.

In this study, foetal bovine serum (FBS) was used as the serum supplement in some of the cell culture media. The FBS originate from USA was purchased from Gibco® and once it arrives in the laboratory, the FBS was stored frozen (-20°C) to preserve the stability of components in the FBS. Since the FBS (Gibco®, Cat. No. 16000-044) purchased was not heat-inactivated, pre-treatment of heat-inactivating the FBS was carried out in the laboratory before supplementing in the culture media.

The frozen FBS was thawed in the refrigerator (3-5°C) to avoid or limit the amount of precipitation. Any precipitate that appears after thawing may be removed by transferring the FBS to sterile tubes and centrifuged (400 x G*)* for 5 minutes. The FBS was heated up to 56°C for 30 minutes to destroy the heat-sensitive complement proteins. The purpose of this is to avoid cell lysis from an immune reaction between the serum proteins and the cells. During the heat-inactivation treatment, the temperature (56°C) and time (30 minutes) was monitored accurately to avoid denaturing other beneficial proteins components in the FBS. The heat-inactivated FBS was cooled to room temperature (25°C) prior to aliquot in sterile conical bottom tubes (50 ml). The FBS aliquots were stored in the freezer (-20°C) with a maximum shelf life of 6 months. Extra frozen-thawed aliquot tubes were stored in the refrigerator (3-5°C) to avoid repeating the freeze/thaw cycle.

3.3.2.8 Preparation of *in vitro* maturation (IVM) medium

Formulation of *in vitro* maturation (IVM) medium in this study was similar for both caprine and bovine oocytes. Fresh IVM medium that consisted of TCM-199 as base medium supplemented with sodium pyruvate (0.2 mM), follicle stimulating hormone (1 µg/ml), oestradiol-17β (1 µg/ml), gentamicin sulphate salt (25 µg/ml) and FBS (10%)

was prepared weekly. For convenient purposes, stock solutions of the supplements were prepared in advance as depicted in Table 3.7.

Table 3.7: Stock solutions for IVM medium

Stock solution (catalogue number)	Concentration (mg/ml)	Method of preparation (Storage temperature and shelf life)
Follicle stimulating hormone, FSH (Folltropin®-V)	5	Folltropin®-V (5 mg) powder was dissolved in solvent (1 ml), aliquot (110 µl) in microcentrifuge tube, sealed with parafilm, wrapped with aluminium foil and stored (-20°C) for 6 months
Oestradiol-17β (E8875)	1	Oestradiol-17β (1 mg) was dissolved in sterile filtered ethanol, GR grade, 95% (1 ml), aliquot in cryovial, sealed with parafilm, wrapped with aluminium foil and stored (3-5°C) for 6 months
Gentamicin sulphate salt (G3632)	50	Gentamicin sulphate salt (1 g) was dissolved in DPBS (20 ml), sterile filtered and aliquot (1 ml) in cryovial, sealed with parafilm, wrapped with aluminium foil and stored (3-5ºC) for 12 months
Sodium pyruvate (P3662)	2.2	Sodium pyruvate (2.2 mg) was dissolved in TCM199 (1 ml), sterile filtered and aliquot in microcentrifuge tube (1.5 ml), sealed with parafilm, wrapped with aluminum foil and stored (3-5ºC) for 2-3 days

Generally, the working solution of the IVM medium (10 ml) was prepared a day prior to use as it required equilibration in a CO_2 incubator (5%) for at least 4 hours. The TCM- 199 (8.9 ml) that served as the basal medium was measured using a disposable plastic pipette (10 ml) and dispensed into a sterile conical tube (15 ml). Subsequently, the L-Cysteine hydrochloride (0.9 mg) was weighed and added into the Tissue Culture Medium-199 (TCM-199). The L-Cysteine hydrochloride dissolved on its own, which

ultimately forms a yellow layer on the bottom of the conical tube. The medium was mixed by gently inverting the conical tube back and forth a few times (5x). Then, other stock solutions such as FSH (10 µl), sodium pyruvate (100 µl), gentamicin (5 µl) and heat-inactivated FBS (1 ml) were added into the conical tube as depicted in Table 3.8. Prior to adding oestradiol-17β, the medium was filter-sterilised using a syringe filter (0.22 µm pore size). The oestradiol-17β stock solution was added at the concentration of 1.0 µl/ml, according to the final volume of the IVM medium after filter. Finally, the IVM working solution was mixed gently before aliquot (5 ml/tube). The aliquot tubes were stored in refrigerator (3-5°C) with the maximum shelf life of 1 week.

Table 3.8: Composition of IVM medium

Chemical component (catalogue number)	Final concentration	Quantity/10 ml
TCM-199 (11150-059)	Basal medium	8.9 ml
L-Cysteine hydrochloride (C7477)	0.57 mM	0.9 mg
FSH stock solution	5 µg/ml	10 µl
Sodium pyruvate stock solution	22 µg/ml	100 µl
Gentamicin stock solution	25 µg/ml	5 µl
FBS (16000-044)	10 %	1 ml
*Oestradiol-17β	1.0 µl/ml	~9.5 µl

(Storage temperature: 3-5°C; shelf life: 1 week)

Note: * Add oestradiol-17β at the concentration of 1.0 µl/ml of the final volume after filter.

3.3.2.9 Preparation of medium for somatic cell line production

The process of producing somatic cell line, in particular adherent cell type and the establishment of cryopreserved somatic cell banking in this study require media such as (a) Tissue culture medium, (b) PBS(-) solution, (c) Trypsin-EDTA solution and (d) Freezing medium.

3.3.2.9 (a) Preparation of tissue culture medium

There are varieties of complete media formulated and available commercially for cell culture. Among them, Eagles's Minimal Essential Medium (MEM) is the commonly used basal medium in tissue culture. In this study, α-MEM, an improved version of the existing MEM formula that contains additional amino acids and vitamins, as well as nucleosides and lipoic acid was used as the basal medium to propagate caprine ear skin fibroblast cell (Freshney, 2000). The nutrients in α-MEM basal medium alone are not sufficient to support cell growth. Thus supplementation of FBS and penicillin G/ streptomycin sulphate salt (PS) into the α-MEM basal medium was done to form a complete tissue culture medium.

(i) Tissue culture medium (stock solution)

The α-MEM (Cat. no.: M0644) stock solution was prepared in advance by dissolving one bottle of α-MEM powder (10.1 g) in Milli-Q water (1 litre). The solution was mixed well using magnetic stirrer (3 minutes) with moderate speed and subsequently, sodium bicarbonate (2.2 g) was weighed and mixed into the α-MEM solution as depicted in Table 3.9. The final mixture of stock solution was filter-sterilised

(0.22 µm pore size) and kept in the refrigerator (3-5°C) for the maximum shelf life of 3 months.

Table 3.9: Composition of α-MEM stock solution

Chemical component (catalogue number)	Final concentration	Quantity/1000 ml
α-MEM (M0644)	Basal medium	10.1 g
$NaHCO_3$ (S5761)	26.2 mM	2.2 g
Milli-Q water	-	1000 ml

(Storage temperature: 3-5°C; shelf life: 3 months)

(ii) Primary explant culture medium (working solution)

Generally, the culture medium for primary explant culture consists of α-MEM stock solution supplemented with FBS (10%) and PS (Table 3.10). In the initial 5 days of primary explant culture, a higher concentration of antibiotics (PS, [3x]) was supplemented into the culture medium to prevent contamination derived from the biopsied tissue. Once the outgrowth of cells was observed, usually from day 5 onwards, the primary explant culture medium was replenished with the tissue culture medium that contain lower concentration of PS [1x]. Reduction in the concentration of antibiotics in the tissue culture medium for the subsequent cell culture is essential to minimise the anti-metabolic effects of the antibiotic that can cross-react with the mammalian cells.

All the primary explant culture media prepared were filter-sterilised using syringe filter (0.22 µm pore size) prior to use. These media were kept in the refrigerator

(3-5°C) with the shelf life of 2 weeks. The culture medium was warmed up in the water bath (37°C) prior feeding the cells.

Table 3.10: Composition of primary cell culture medium

Chemical component (catalogue number)	Final concentration	Quantity/100 ml
α-MEM stock	-	90 ml
FBS (16000-044)	10%	10 ml
*PS stock [100x]	3x or 1x	300 µl or 100 µl

(Storage temperature: 3-5°C; shelf life: 2 weeks)
Note: *PS [3x] for first 5 days of primary explant culture, PS [1x] from day 5 to 9.

(iii) Cell line culture medium (working solution)

The culture medium for cell line culture consisted of similar tissue culture medium composition mentioned above with the concentration of antibiotic (PS, [1x]) as depicted in Table 3.11. The cell line culture medium was filter-sterilised using syringe filter (0.22 µm pore size) and kept in the refrigerator (3-5°C) with the shelf life of 2 weeks. Prior to use, the culture medium was warmed up in the water bath (37°C).

Table 3.11: Composition of cell line culture medium

Chemical component (catalogue number)	Final concentration	Quantity/100 ml
α-MEM stock	-	90 ml
FBS (16000-044)	10%	10 ml
PS stock [100x]	1x	100 µl

(Storage temperature: 3-5°C; shelf life: 2 weeks)

3.3.2.9 (b) Preparation of PBS(-)

In tissue culture work, phosphate-buffered saline solution without calcium chloride and magnesium chloride (PBS(-)) was used for washing and suspending cells. In an adherent monolayer tissue culture system, calcium and magnesium ions are known to promote cell to cell and cell to substrate binding. In order to facilitate the disaggregation of cells during trypsinisation, the adherent cells were washed with PBS(-) prior to trypsinisation. This was done with the purpose of reducing the concentration of divalent cations and traces of FBS proteins that present in the tissue culture medium which would inhibit trypsin action. The PBS(-) was prepared according to the chemical composition depicted in Table 3.3.

3.3.2.9 (c) Preparation of trypsin-EDTA solution

The caprine ear skin fibroblast primary culture and cell line culture were propagated as an adherent monolayer. Thus, the attachment of cells to each other and to the culture substrate is mediated by cell surface glycoproteins and Ca^{2+}. Other proteins and

proteoglycans derived from the cells and from the serum supplemented become associated with the cell surface and the surface of the substrate and facilitate cell adhesion. Hence, in order to dissociate cells from the substrate and to generate single cell suspension during subculture or cell harvesting, digestive enzyme that breakdown peptide bond and Ca^{2+} chelator are required. In this study, enzyme trypsin (0.25%) supplemented with ethylenediaminetetraacetic acid (EDTA) that chelates Ca^{2+} was used.

Trypsin (0.5 g) was first dissolved in the PBS(-) solution (200 ml) and subsequently, EDTA (0.08 g) was added. The solution was mixed well using magnetic stirrer before adding penicillin G/streptomycin sulphate (PS) stock (200 µl) (Table 3.12). The final solution was filter-sterilised using syringe filter (0.22 µm pore size) before kept in the refrigerator (3-5°C) for future use with the shelf life of 1 month.

Table 3.12: Composition of trypsin-EDTA solution

Chemical component (catalogue number)	Final concentration	Quantity/200 ml
Trypsin (T4799)	0.25%	0.5 g
EDTA (E9884)	1.37 mM	0.08 g
PBS(-) solution	-	200 ml
PS stock [100x]	1x	200 µl

(Storage temperature: 3-5°C; shelf life: 1 month)

3.3.2.9 (d) Preparation of freezing medium

Cryopreservation is an important process in tissue culture whereby cells or whole tissues are preserved by cooling to low sub-zero temperatures such as -196°C in the liquid nitrogen for long term storage purposes. Generally, the cryopreservation medium or freezing medium is formulated to contain cryoprotectants. Cryoprotectants such as ethylene glycol, dimethyl sulfoxide (DMSO), glycerol, and sucrose could help to reduce cryo-injuries on the cells due to extracellular ice formation, dehydration and intracellular ice formation.

In this study, freezing medium was prepared by supplementing DMSO (10%) into the tissue culture medium used for cell line propagation (Table 3.13). The freezing medium was prepared fresh on the day of cryopreservation with the amount just sufficiently to cater the amount of cell to be cryopreserved. Preparation of freezing medium in mass volume is not encouraged as the shelf life is short (5 days) kept in the refrigerator (3-5°C).

Table 3.13: Composition of freezing medium

Chemical component (catalogue number)	Final concentration	Quantity/50 ml
Tissue culture medium (working solution + PS [1x])	-	45 ml
DMSO (D5879)	10%	5 ml

(Storage temperature: 3-5°C; shelf life: 5 days)

3.3.2.10 Preparation SCNT manipulation media

The general SCNT procedure involves three steps: enucleation of the oocyte (recipient cytoplast), insertion of donor cell (via sub-zonal injection followed by electrofusion) and the activation of reconstructed embryo. A series of media such as hyaluronidase solution, oocyte holding solution at normal air environment (EmCare®), Cytochalasin B, incubated-oocyte holding solution (used in 38.5°C, 5% CO_2), fusion medium and activation chemical are required on the day of experiment to reconstruct the embryo according to the three main steps mentioned above.

3.3.2.10 (a) Preparation of hyaluronidase solution

Hyaluronidase is an enzyme used to denude or remove cumulus cells from COCs after *in vitro* maturation in this study. The hyaluronidase solution (0.2%) was prepared by dissolving bovine testes-derived hyaluronidase powder (0.1 g) in mDPBS with PVP-360 solution (50 ml) as depicted in Table 3.14. The prepared solution was filter-sterilised using syringe filter (0.22 µm pore size), aliquot (150 µl) in microcentrifuge tubes and stored for 6 months in freezer (-20°C).

Table 3.14: Composition of hyaluronidase solution

Chemical component (catalogue number)	Final concentration	Quantity/50 ml
Hyaluronidase (H4272)	0.2%	0.1 g
PVP-360	0.1%	0.05 g
mDPBS	1x	50 ml

(Storage temperature: -20°C; shelf life: 6 months)

3.3.2.10 (b) Oocyte holding solution at normal air environment

Commercially available EMCARE^{TM} holding solution manufactured by ICPbio Reproduction, New Zealand was used as a holding solution for oocyte and embryo at normal air environment. According to the description adapted from the product factsheet, this solution consist of optimised nutritional composition that enhances embryo development, MOPS buffer, New Zealand-origin bovine albumin that serve as protective surfactant and energy source, as well as kanamycin sulphate antibiotic.

The EMCARE^{TM} holding solution was aliquot (10 ml) in conical tube (15 ml) and stored in the refrigerator (3-5°C) for 6 months or according to the expiry date printed on the product label. The oocyte holding solution was warmed in the water bath (37°C) prior to use.

3.3.2.10 (c) Preparation of cytochalasin B solution

Cytochalasin B (CB) is a cell-permeable mycotoxin isolated from *Drechslera dematoidea*. It acts as an inhibitor of microfilament formation by preventing actin

polymerisation. Thus it was used in the oocyte enucleation step as a cytoskeletal relaxant agent to prevent ooplasm lysis and damage during this procedure.

(i) Cytochalasin B stock solution

The CB stock solution was prepared in advance by dissolving CB powder (1 mg) in DMSO (1 ml) as depicted in Table 3.15. The stock solution was mixed well by gently pipetting in and out before aliquot (10 µl) into microcentrifuge tubes (1.5 ml). The aliquot tubes were wrapped with aluminium foil before keeping in the freezer (-20°C) with the shelf life of 6 months.

Table 3.15: Composition of cytochalasin B stock solution

Chemical component (catalogue number)	Final concentration	Quantity/1 ml
Cytochalasin B (C6762)	1 mg/ml	0.001 g
DMSO (D5879)	-	1 ml

(Storage temperature: -20°C; shelf life: 6 months)

(ii) Cytochalasin B working solution

The CB working solution was prepared by diluting the defrosted stock solution (10 µl) with EmCare®, oocyte holding solution (990 µl) as shown in Table 3.16. This procedure was carried out just before the preparation of micromanipulation dish.

Table 3.16: Composition of cytochalasin B working solution

Chemical component (catalogue number)	Final concentration	Quantity/1 ml
Cytochalasin B stock solution	10 µg/ml	10 µl
EmCare® solution	-	990 µl

3.3.2.10 (d) Preparation of incubated-oocyte holding solution

In order to avoid the exposure of oocytes to the room temperature too long during the SCNT manipulation procedure, batches of manipulated oocytes were kept in the incubator (38.5°C, 5% CO_2 in air) at the interval of conducting the oocyte enucleation, donor cell insertion and activation of reconstructed embryo procedure.

TCM-199 supplemented with FBS (10%) was used as the oocyte holding solution (Table 3.17) under the incubated environment (38.5°C, 5% CO_2 in air). The incubated-oocyte holding solution was filter-sterilised using syringe filter (0.22 µm pore size). Prior to use, the incubated-oocyte holding solution was equilibrated at least 3 hours in the incubator (38.5°C, 5% CO_2 in air).

Table 3.17: Composition of incubated-oocyte holding solution

Chemical component (catalogue number)	Final concentration	Quantity/5 ml
TCM-199	-	4.5 ml
FBS (16000-044)	10%	0.5 ml

(Storage temperature: 3-5°C; shelf life: 1 week)

3.3.2.10 (e) Preparation of fusion solution

The donor cell (donor karyoplast) was introduced into the enucleated oocyte (recipient cytoplast) via sub-zonal injection followed by fusion. Fusion of the karyoplast-cytoplast couplets were accomplished by electrical stimulation in a medium that is non-ionic and slightly hypotonic. In this study, Zimmermann's fusion medium (ZFM) formulated by Zimmermann and Vienken (1982) was used. ZFM was prepared according to the chemical composition depicted in Table 3.18. The ZFM was filter-sterilised using syringe filter (0.22 µm pore size) and kept in the refrigerator (3-5°C) with the shelf life of 3 months. Prior to use, the ZFM solution was warmed to 37°C in the water bath.

Table 3.18: Composition of ZFM solution

Chemical component (catalogue number)	Final concentration	Quantity/125 ml
Sucrose (S7903)	0.28 mM	11.98 g
$Mg(C_2H_3O_2)_2.4H_2O$ (M0631)	0.5 mM	0.0134 g
$Ca(C_2H_3O_2)_2$ (C4705)	0.1 mM	0.002 g
K_2HPO_4 (P3786)	1.0 mM	0.0218 g
L-Glutathione (G4251)	0.1 mM	0.0039 g
BSA-V (A6003)	0.001%	0.0013 g
Milli-Q	-	125 ml

(Storage temperature: 3-5°C; shelf life: 3 months)

3.3.2.10 (f) Preparation of activation medium

Oocyte activation is a process that occurs in order to enable oocyte exit from the MII arrest. In physiological condition, sperm entry initiates oocyte activation by inducing intracellular calcium (Ca^{2+}) oscillation. After a few hours, a series of biochemical events make fertilisation complete, through the inactivation of maturation promoting factor (MPF) and of mitogen-activated protein kinase (MAPk) that leads to resumption and completion of meiosis, DNA synthesis and pronuclei formation. However, in nuclear transfer (in the absence of sperm), artificial oocyte activation procedure that closely

mimic normal fertilisation such as mechanical, chemical or physical stimuli that elicit one or several Ca^{2+} transients are required. In this study, chemical activation procedure was incorporated. There are several chemical that could be used as activation agents such as calcium ionophore (CaI), Ionomycin, 7% ethanol (EtOH), cycloheximide (CHX), cytochalasin D (CD) and strontium chloride (SrCl). Some of these activation chemicals can be used singly or in combinations of two or more. Two chemical activation protocols were used in this study namely:

- Protocol A: EtOH [7%] (5 min) followed with CD [1.25 µg/ml] + CHX [10 µg/ml] (5 h); for gaur interspSCNT, caprine interspSCNT, bovine intraspSCNT and PA embryos production
- Protocol B: CaI [5 µM] (5 min) followed with 6-DMAP [2 mM] (4 h), developed by Shen *et al*. (2008); for caprine intraspSCNT, interspSCNT and PA embryos production

(i) Preparation of ethanol solution (7%)

Ethanol (EtOH; 7%), is one of the oocyte activation agents that promotes the formation of inositol 1,4,5-trisphosphate (IP_3) and the influx of extracellular Ca^{2+}. The exposure of matured oocytes to EtOH (7%) is reported to be (5-7 minutes) effective in inducing successful activation and pronuclear formation (Presicce and Yang, 1994). The EtOH (7%) was prepared by diluting absolute EtOH (70 µl) with oocyte holding solution, EmCare® (930 µl) as depicted in Table 3.19. The EmCare® solution was pre-warmed in a water bath (37°C) before diluting the absolute EtOH,

Table 3.19: Composition of EtOH (7%) solution

Chemical component (catalogue number)	Final concentration	Quantity/ 1ml
Absolute EtOH	7%	70 µl
EmCare®	-	930 µl

EtOH: Ethanol.

(ii) Preparation of cytochalasin D solution

Cytochalasin D is a cell permeable fungal toxin that inhibits actin polymerisation. Thus, it may function to prevent the release of second polar body after activation of the matured oocytes by disrupting the spindle formation (Sun *et al*., 2001).

(ii.a) Preparation of cytochalasin D stock solution

The initial CD stock solution (1st stock) with the concentration of 1 mg/ml was prepared by dissolving CD powder (1 mg) with DMSO (1 ml) as shown in Table 3.20. A serial dilution of this CD stock solution to the concentration of 0.1 mg/ml (2nd stock) was done by mixing the CD 1st stock solution (0.5 ml) with TCM-199 (5 ml) as depicted in Table 3.21. The CD 2nd stock solution was aliquot (12.5 µl) into microcentrifuge tubes (1.5 ml) wrapped with aluminium foil and stored in the freezer (-20°C) with the shelf life of 3 months.

Table 3.20: Composition of cytochalasin D 1st stock solution

Chemical component (catalogue number)	Final concentration	Quantity/ 1ml
Cytochalasin D (C8273)	1 mg/ml	0.001 g
DMSO (D5879)	-	1 ml

(Storage temperature: -20°C; shelf life: 6 months)

Table 3.21: Composition of cytochalasin D 2nd stock solution

Chemical component (catalogue number)	Final concentration	Quantity/ 5 ml
CD (1st stock) (C8273)	0.1 mg/ml	0.5 ml
TCM-199 (11150-059)	-	4.5 ml

(Storage temperature: -20°C; shelf life: 3 months)
CD: cytochalasin D.

(iii) Preparation of cycloheximide solution

Cycloheximide (CHX) is an antibiotic produced by *Streptomycin griseus*. CHX is known to be a protein synthesis inhibitor that activates mammalian oocytes by blocking the continuous synthesis of cyclin B that is required to stimulate the cell cycle protein kinase CDK 1 (Moses and Kline, 1995).

(iii.a) Preparation of cycloheximide stock solution

The CHX stock solution was prepared by dissolving CHX powder (5 mg) in TCM-199 solution (5 ml) as depicted in Table 3.22. The stock solution was aliquot (10 µl) into

microcentrifuge tubes (1 ml) wrapped with aluminium foil and stored in the freezer (-20°C) with the shelf life of 3 months.

Table 3.22: Composition of cycloheximide stock solution

Chemical component (catalogue number)	Final concentration	Quantity/ 5 ml
Cycloheximide (C7698)	1 mg/ ml	0.005 g
TCM-199 (11150-059)	-	5 ml

(Storage temperature: -20°C; shelf life: 3 months)

(iv) Preparation of cytochalasin D – cycloheximide working solution

The CD-CHX working solution was prepared fresh on the day of experiment by mixing the CD 2nd stock solution and CHX stock solution with IVC medium. This activation protocol was used in gaur interspSCNT experiment, thus the IVC medium that was used to dilute the CD-CHX stock was modified synthetic oviductal fluid (mSOF) as depicted in Table 3.23. Prior to use, CD-CHX solution was equilibrated in the incubator (5% CO_2, 38.5°C) for a least 4 hours.

Table 3.23: Composition of CD- CHX working solution

Chemical component (catalogue number)	Final concentration (µg/ml)	Quantity (µl)
CD (2nd stock) solution	1.25	12.5
CHX stock solution	10.0	10.0
IVC medium	-	977.5

(v) Preparation of calcium ionophore solution

Calcium ionophore (CaI) promotes the release of intracellular Ca^{2+} and also facilitates the influx of extracellular Ca^{2+} (Kline and Kline, 1992).

(v.a) Calcium ionophore stock solution

The calcium ionophore (CaI) stock solution [500 µM] was prepared by dissolving CaI powder (0.001 g) in DMSO (3.82 ml) as depicted in Table 3.24. The CaI stock solution was aliquot (10 µl) in microcentrifuge tubes (1.5 ml), wrapped with aluminium foil and kept in the freezer (-20°C) for the shelf life of 3 months.

Table 3.24: Composition of calcium ionophore stock solution

Chemical component (catalogue number)	Final concentration	Quantity
Calcium ionophore (C7522)	500 µM	0.001 g
DMSO (D5879)	-	3.82 ml

(Storage temperature: -20°C; shelf life: 3 months)

(v.b) Calcium ionophore working solution

The CaI stock solution (10 µl) was defrosted in room temperature prior subjected to dilution with IVC medium (990 µl) as depicted in Table 3.25. The final concentration of CaI working solution was 5 µM and this working solution must be equilibrated in the incubator (38.5°C, 5% CO_2 in air) at least 4 hours prior to use.

Table 3.25: Composition of calcium ionophore working solution

Chemical component (catalogue number)	Final concentration	Quantity/1 ml
Calcium ionophore stock solution	5 µM	10 µl
IVC medium	-	990 µl

(vi) Preparation of 6-dimethylaminopurine (6-DMAP) solution

6-dimethylaminopurine (6-DMAP) is a kinase inhibitor that was usually used after a brief exposure of the oocytes to CaI, in order to generate a persistent kinase inactivation.

(vi.a) 6-dimethylaminopurine (6-DMAP) stock solution

6-DMAP stock solution [0.2 M] was prepared by dissolving 6-DMAP powder (0.1 g) in Milli-Q water (3.08 ml) as shown in Table 3.26. The stock solution was aliquot (10 µl) in microcentrifuge tubes (1.5 ml), wrapped with aluminium foil and stored in the freezer (-20°C) with the shelf life of 6 months.

Table 3.26: Composition of 6-DMAP stock solution

Chemical component (catalogue number)	Final concentration	Quantity
6-DMAP (D2629)	0.2 M	0.1 g
Milli-Q water	-	3.08 ml

(Storage temperature: -20°C; shelf life: 6 months)
6-DMAP: 6-dimethylaminopurine.

(vi.b) 6-dimethylaminopurine working solution

The 6-DMAP stock solution (10 µl) was defrosted at room temperature (25°C) prior subjected to dilution with IVC medium (990 µl) as depicted in Table 3.27. The final concentration of 6-DMAP working solution was 2 mM and this working solution must be equilibrated in the incubator (38.5°C, 5% CO_2 in air) at least 4 hours prior to use.

Table 3.27: Composition of 6-DMAP working solution

Chemical component (catalogue number)	Final concentration	Quantity/ 1ml
6-DMAP stock solution	2 mM	10 µl
IVC medium	-	990 µl

6-DMAP: 6-dimethylaminopurine.

3.3.2.11 Preparation of *in vitro* culture medium

There were two different *in vitro* culture (IVC) media used in this study that incorporate either the basal medium of modified synthetic oviduct fluid (mSOF) or potassium simplex optimisation medium with amino acid (KSOMaa). The mSOF medium was used to culture both gaur, bovine and caprine cloned embryos, while KSOMaa was used only in the caprine intraspSCNT and interspSCNT study.

3.3.2.11(a) Preparation of modified synthetic oviduct fluid medium

Synthetic oviduct fluid (SOF) medium was originally formulated by Tervit *et al*. (1972) and further modification of this medium (mSOF) was done by Takahashi and First

(1992). As culture medium need to be freshly prepared weekly or biweekly with accurate but time consuming measurements, hence stock solutions in a concentrated form (10x) was prepared for the convenient purpose of the subsequent working solution (1x) preparation procedure (Nagy *et al*., 2003).

(i) Preparation of modified synthetic oviduct fluid stock solution

All the components, except phenol red were weighed accordingly to the measurements depicted in Table 3.28 using a digital balance and contained in a measuring cylinder (100 ml). Ultrapure Milli-Q water (approximately 40 ml) was added to dissolve the chemical components and followed by the addition of phenol red (0.5%) solution. The entire solution was mixed well using the magnetic stirrer at a moderate speed. Then ultrapure water was added again to make the final volume (50 ml). The resulting mSOF stock medium was filter-sterilised using syringe filter (0.22 µm pore size), stored in screw cap bottle (50 ml) for a shelf life of 1 month.

Table 3.28: Composition of modified synthetic oviduct fluid stock solution [10x]

Chemical component (catalogue number)	Concentration at 1x (mM)	Quantity/50 ml
NaCl (S5886)	107.70	3.1470 g
KCl (P5405)	7.16	0.2669 g
KH_2PO_4 (P5655)	1.19	0.0810 g
Phenol red, 0.5% (P0290)	-	500 µl
$CaCl_2.2H_2O$ (C7902)	1.71	0.1257 g
$MgCl_2.6H_2O$ (M2393)	0.49	0.0498 g
Milli-Q water	-	~49.50 ml

(Storage temperature: 3-5°C; shelf life: 1 month)

(ii) Preparation of modified synthetic oviduct fluid working solution

The mSOF working solution at 1x concentration was prepared by supplementing mSOF stock solution [10x] with chemical components as depicted in Table 3.29. The mSOF stock solution (5 ml) was dispensed into a measuring cylinder (100 ml). Subsequently, Milli-Q water (20 ml) was added into the measuring cylinder. The sodium pyruvate, L-glutamine, sodium bicarbonate were weighed and added into the solution. Subsequently, sodium lactate syrup, BME and MEM were added into the solution using micropipette. The mSOF working solution was top up with Milli-Q water to the final volume of 50 ml. The entire solution was mixed well using magnetic stirrer at a moderate speed prior subjecting to filter-sterilisation (0.22 µm pore size). The resulting mSOF working solution was kept in screw cap bottle (100 ml) with shelf life of 1 month in the refrigerator (3-5°C).

Table 3.29: Composition of modified synthetic oviduct fluid working solution

Chemical component (catalogue number)	Final concentration	Quantity/50 ml
mSOF stock solution [10x]	1x	5 ml
Sodium pyruvate (P4562)	0.33 mM	0.0018 g
L-Glutamine (G3126)	1 mM	0.0073 g
$NaHCO_3$ (S5761)	25.1 mM	0.1053 g
Na-Lactate (L7900)	2.83 mM	23.5 µl
BME amino acids solution [50x] (B6766)	1x	1 ml
MEM non-essential amino acids solution [100x] (M7145)	1x	0.5 ml
Milli-Q	-	~43.48 ml
PS stock [100x]	1x	50 µl

(Storage temperature: 3-5°C; shelf life: 1 month)

The final IVC medium was prepared by adding BSA-V (0.003 g) into the mSOF working solution as depicted in Table 3.30. The final IVC medium was kept in conical tube (15 ml) with the shelf life of 1 week in the refrigerator (3-5°C). Prior to use, the IVC medium was equilibrated in the incubator (5% CO_2, 38.5°C) for a least 4 hours by loosening the cap of the tube.

Table 3.30: Composition of *in vitro* culture medium-mSOF base

Chemical component (catalogue number)	Final concentration	Quantity/ 10 ml
mSOF working solution	1x	10 ml
BSA-V (A6003)	0.3%	0.03 g

(Storage temperature: 3-5°C; shelf life: 1 week)

3.3.2.11(b) Preparation of potassium simplex optimisation medium with amino acid

Potassium simplex optimisation medium (KSOM) was originally formulated by Lawits and Biggers (1993) using a computer model. Further modification on this medium was done with the supplementation of amino acids (KSOMaa) by Biggers *et al*. (2000). In this study, two types of KSOMaa base culture media were used:

(a) KSOMaa A- classical/standard KSOMaa

(b) KSOMaa B- KSOMaa supplemented with glucose (2.78 mM final concentration)

The KSOMaa B was a modification of KSOMaa and the effect of supplementation of glucose in KSOMaa B was part of the experimental design to improve the blastocyst rate of the caprine cloned embryos.

(i) Preparation of potassium simplex optimisation medium with amino acid stock solution

In each batch of KSOMaa stock solution preparation, usually 100 ml of the medium was prepared. All the chemical components in powdered form were weighed and added into a measuring cylinder contained Milli-Q water (50 ml) according to the sequence shown in Table 3.31. The sodium lactate (0.186 ml) was added into the solution (50 ml) using a micropipette (200 µl). Then the entire solution was mixed well using magnetic stirrer at a moderate speed before filter-sterilised (0.22 µm pore size). The resulting KSOMaa stock solution was dispensed into a screw cap bottle (100 ml) and kept in the refrigerator (3-5°C) with the shelf life of 1 month.

Table 3.31: Composition of KSOMaa stock solution

Chemical component (catalogue number)	Final concentration (mM)	Quantity/100 ml
NaCl (S5886)	95.0	0.5553 g
KCl (P5405)	2.50	0.0186 g
KH_2PO_4 (P5655)	0.35	0.0048 g
$MgSO_4$ (M7506)	0.20	0.0024 g
Na Lactate, 60% syrup (L7900)	10.0	0.1860 ml
Sodium pyruvate (P4562)	0.20	0.0022 g
D-Glucose (G6152)	0.20	0.0036 g
$NaHCO_3$ (S5761)	25.0	0.2101 g
$CaCl_2$ (C5670)	1.71	0.0190 g
L-Glutamine (G3126)	1.0	0.0146 g
EDTA (E9884)	0.01	0.0004 g
Milli-Q water	-	99.814 ml

(Storage temperature: 3-5°C; shelf life: 1 month)

(ii) Preparation of potassium simplex optimisation medium with amino acid working solution

There were two types of KSOMaa base-IVC medium used in this study, which namely:

(a) KSOMaa A- classical/standard KSOMaa

(b) KSOMaa B- KSOMaa supplemented with glucose (2.78 mM final concentration)

KSOMaa A and B were used as the IVC media in the two-step culture system of the cloned caprine embryos. The chemical composition of KSOMaa A was depicted in Table 3.32. KSOMaa A and 20 ml of the medium was prepared each time.

KSOMaa B was prepared by supplementing KSOMaa A (10 ml) with glucose (0.0046 g) at the final concentration of 2.78 mM (Table 3.33). Using only one syringe-filter (0.22 µm pore size), the resulting KSOMaa A was first filter-sterilised followed by KSOMaa B. Both media was kept in the refrigerator (3-5°C) with the shelf-life of 1 week. Prior to use, the IVC medium was equilibrated in the incubator (5% CO_2, 38.5°C) for a least 4 hours by loosening the cap of the tube.

Table 3.32: Composition of *in vitro* culture medium-KSOMaa A

Chemical component (catalogue number)	Final concentration	Quantity/ 20 ml
KSOMaa stock solution	1x	20 ml
BSA-V (A6003)	0.4%	0.08 g
MEM non-essential amino acids solution [100x] (M7145)	-	100 µl
BME amino acids solution [50x] (B6766)	-	200 µl

(Storage temperature: 3-5°C; shelf life: 1 week)

Table 3.33: Composition of *in vitro* culture medium-KSOMaa B

Chemical component (catalogue number)	Final concentration	Quantity/ 10 ml
KSOMaa A solution	1x	10 ml
D-Glucose (G6152)	2.78 mM	0.0046 g

(Storage temperature: 3-5°C; shelf life: 1 week)

3.3.2.12 Preparation of nucleic acid staining solution

In this present study, nucleic acid staining was conducted to determine the location of first polar body and oocyte nucleus of matured caprine oocyte subjected to *in vitro* maturation (IVM) at different durations and to determine the total cell number in the cloned and parthenogenesis blastocysts. In order to carry out the nucleic acid staining, fixative solution and Hoechst 33342 were prepared.

Fixative solution was used to preserve the biological material or morphology of the oocyte and blastocyst after staining. For the ease of observation and evaluation, the stained specimens were kept for several weeks in order to view them under the fluorescent microscope in one session.

Hoechst 33342 or bisBenzimide was used to stain the oocytes and blastocyst after fixation. Hoechst 33342 is a blue fluorescent dye that used to stain DNA. This dye bind to the minor groove of double stranded DNA with a preference for sequences rich in adenine and thymine. Hoechst 33342 dye is cell-permeable and can bind to DNA in live or fixed cells.

3.3.2.12 (a) Preparation of fixative solution

Typically, the fixative solution was prepared by mixing PBS(-) with formaldehye, PVP-40 and glutaraldehyde according to the sequence and concentration shown in Table 3.34. The PVP-360 was left to dissolve on its own without any stirring motion to avoid foam formation. After the PVP-40 was completely dissolved, the solution was mixed by gently inverting the conical tube (15 ml) containing the solution back and forth a few times (5x). The resulted fixative solution in the conical tube was wrapped with aluminium foil and stored in refrigerator (3-5°C) for the shelf life of 6 months.

Table 3.34: Composition of fixative solution

Chemical component (catalogue number)	Final concentration	Quantity/ 10 ml
PBS(-) solution	1x	~9.78 ml
Formaldehyde (40%) (F8775)	0.8%	200 µl
Polyvinylpyrolidone-40 (Sigma PVP-40)	0.1%	0.01 g
Glutaraldehyde (25%) (G5882)	0.06%	25 µl

(Storage temperature: 3-5°C; shelf life: 6 months)

3.3.2.12 (b) Preparation of Hoechst 33342 dye

Hoechst 33342 stock solution was prepared according to the chemical composition depicted in Table 3.35. The Hoechst 33342 powder was dissolved in PBS(-) by gently inverting the conical tube (15 ml) containing the solution back and forth a few times (10x). The resulting Hoechst 33342 stock solution was aliquot (100 µl) in microcentrifuge tube (1.5 ml), wrapped with aluminum foil and kept in the refrigerator (3-5°C) for the shelf life of 6 months.

Table 3.35: Composition of Hoechst 33342 stock solution

Chemical component (catalogue number)	Final concentration	Quantity/ 10 ml
PBS(-)	1x	10 ml
Hoechst 33342 (B2261)	0.025%	0.0025 g

(Storage temperature: 3-5°C; shelf life: 6 months)

After the preparation of Hoechst 33342 stock solution, Hoechst 33342 working solution was prepared by adding Hoechst 33342 stock solution (100 µl) with PBS(-) (900 µl) in a conical tube (15 ml) using micropipette. The solution was mixed well before supplemented with glycerol (1 ml) as depicted in Table 3.36. The working solution was aliquot (1 ml) into microcentrifuge tubes (1.5 ml), wrapped with aluminium foil and kept in the refrigerator (3-5°C) for the shelf life of 6 months.

Table 3.36: Composition of Hoechst 33342 working solution

Chemical component (catalogue number)	Final concentration	Quantity/ 2 ml
Hoechst 33342 stock solution	0.0025%	0.1 ml
PBS(-) solution	1x	0.9 ml
Glycerol (G2025)	-	1 ml

(Storage temperature: 3-5°C; shelf life: 6 months)

3.3.3 Preparation of Mouth pipette assembly

Mouth pipette assembly is a device used for all oocyte and embryo handling in this study. The mouth pipette assembly used in this study was prepared using a 200 µl tip (as an aspirator mouthpiece), silicone tubing, a syringe filter (0.22 µm pore size), a 1000 µl

tip (as Pasteur pipette holder) and a Pasteur pipette pulled on a flame to create a narrow opening (Figure 3.1).

Generally, when using the mouth-controlled pipette for oocytes or embryo handling, clean medium was first aspirated into the capillary before picking up embryos. This was an important precaution taken to reduce the capillary action that draws the embryos into the pipette and may causes embryo losses. It also helps to avoid accidental expelling of air bubbles when embryos were released from the capillary.

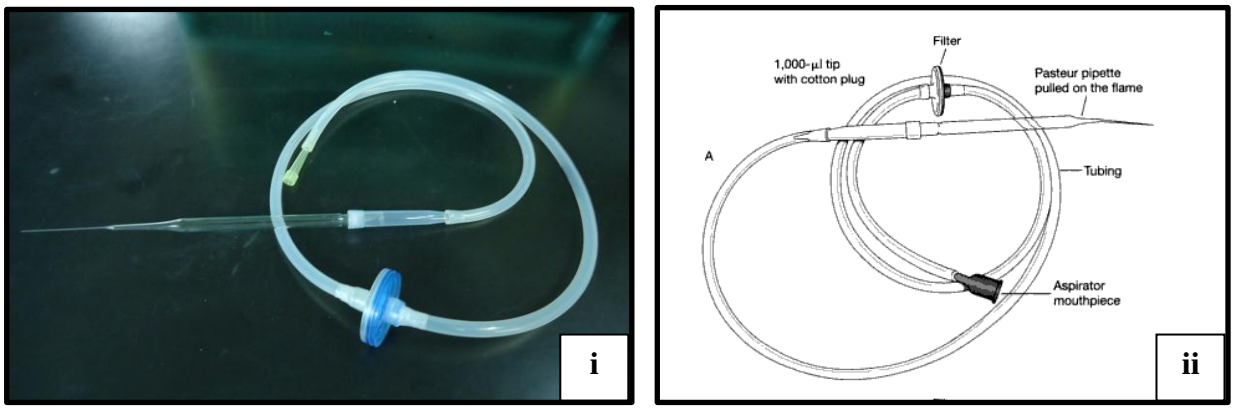


Figure 3.1: Preparation of mouth pipette assembly. (i) Original photograph; (ii) Labelled photograph (adapted from Nagy *et al*., 2003).

3.3.3.1 Cleaning and sterilisation of Pasteur pipette

The glass Pasteur pipette were rinsed thoroughly with RO water (3 times) before soaking overnight in the RO water. After 24 hours, the Pasteur pipettes were rinsed with Milli-Q water (5 times) before heat drying in the oven (56°C) for 1 to 2 days. The dried Pasteur pipettes were packed in the autoclave bag before sterilising in the autoclave machine (20 to 25 minutes at 121°C, 15 psi). The sterile Pasteur pipettes were dried in the oven before using.

3.3.3.2 Preparation of Mouthpiece-controlled pipette

The mouthpiece-controlled pipette was prepared by narrowing the inner diameter of the existing glass Pasteur pipette to the appropriate diameter (160 to 180 µm for oocyte and embryos; 330 to 350 µm for COC). The tapered section of the Pasteur pipette was subjected to a fine spirit burner flame in a rotating motion until the glass softened (Figure 3.2). Once the glass softened, the Pasteur pipette was immediately withdrawn from the heat and in a speed second, both ends of the Pasteur pipette were pulled smoothly to the opposite direction. Both ends were held steady for a few seconds, until the capillary segment has cooled and the capillary was broken by a sharp pull in the opposite directions. The thinned portion of the Pasteur pipette was scribed with a diamond stone at the point in which the diameter suits the need of the usage. Subsequently, the scribe point was snapped gently and the diameter of the resulting mouthpiece-controlled pipette was examined under the microscope. Prior to use, the tip of the mouthpiece-controlled pipette was fire-polished by a quick touch of the tip to the flame.

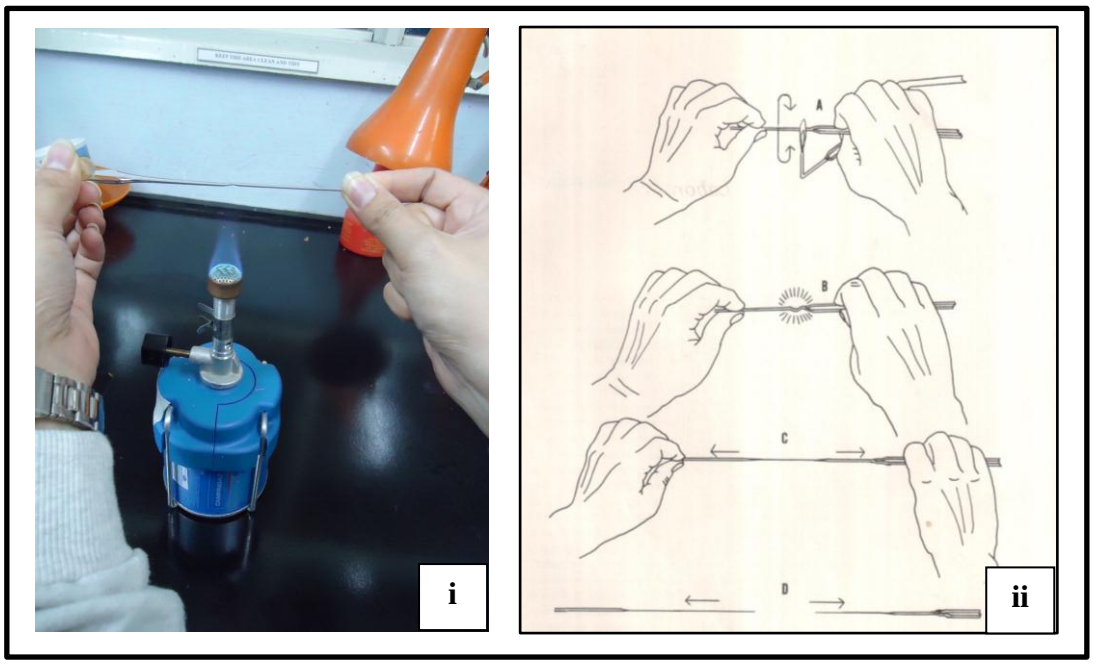


Figure 3.2: Preparation of mouthpiece-controlled pipette. (i) Original photograph; (ii) procedure for drawing micropipette (adapted from Rafferty, 1970).

3.3.4 Preparation of microtools for SCNT manipulation

Microtools such as holding pipette, enucleation needle and injection pipette were prepared in-house for SCNT manipulation. The microtools were prepared using thin-walled borosilicate glass capillaries (inner diameter: 0.69 µm; outer diameter: 0.97 µm; length 10 cm) fabricated with three main instruments namely, horizontal micropipette puller, microforge and microgrinder (Figure 3.3).

Horizontal micropipette puller (P-97, Sutter Instrument, USA) was used to pull the borosilicate glass capillaries to form pipette with micro diameter tip. The length and diameter of the micropipette generated was controlled by adjusting a set of parameter that determines pulling mechanism of the machine shown in Table 3.37.

Table 3.37: Description of the micropipette puller's parameter

Parameter	Characteristics
Heat	• Heat controls the level of electrical current supplied to the filament that melt the glass. • Higher heat setting give longer and finer tips
Pull strength	• Controls the force of the hard pull • Higher pull, the smaller the pipette's tip diameter and longer its taper • Injection needle with large tip (40-75) • Micropipette with smaller tip (120-250)
Velocity	• The velocity value determines the point of which the heat is turned off • Micropipette: 80-120 • Microinjection: 50-80
Pressure	• The pressure of the cooling air delivered to the filament • Higher the pressure, the shorter the pipette taper • Thin wall glass: ≤ 300 unit • Thick wall: 500 unit
Time	• This parameter control the length of the cooling air is active (1 unit of time = 1/2 µsec)

(Summarised from material adapted from: http://www.sutter.com/manuals/P-97-DOM_OpMan.pdf)

In this present study, the set of parameter (heat= 665 units, pull=150 units, velocity= 100, time= 150 units, pressure= 500 units) was used to shape the microneedle that has a long and uniform tapering end with a length of approximately 10 mm. The shape of the microneedle tip was further refined using microforge and microgrinder.

Microforge (Technical Products Internationals, USA) comprised of a horizontal microscope combined with fine adjustment filament, tool holder and an electrical air blower. This device is used to cut, shape, polish and bend the microneedle for the preparation of holding pipette, cutting needle and injection needle. Prior using this device to fabricate any microtools, a glass bead was made on top of the filament. In order to prepare the glass bead, the filament and microneedle were aligned vertically and the position was adjusted until a clear focus was observed under the microscope. The microneedle was raised above the filament before heating up the filament. Once the filament was heated until a red glow observed on it (heat control level= 4 to 5), the microneedle was lowered down gradually touching the filament. To shape the melted borosilicate glass into a ball shape, the microneedle was repeatedly pulled up and down until the glass bead with the diameter of 20 to 30 µm was obtained. The heat control power was switched off and the microneedle was detached from the glass bead by snapping the fine tip of the microneedle with a forceps. Once again, the glass bead was heated gradually to polish the bead surface.

Microgrinder (EG-4, Narashige Co. Ltd., Japan) was used to bevel the tip of the micropipette. This device comprises of a motorised grinder plane, pipette holder and a protractor mounted on the pipette holder fixing section to facilitate the adjustment of the required grinding angle.

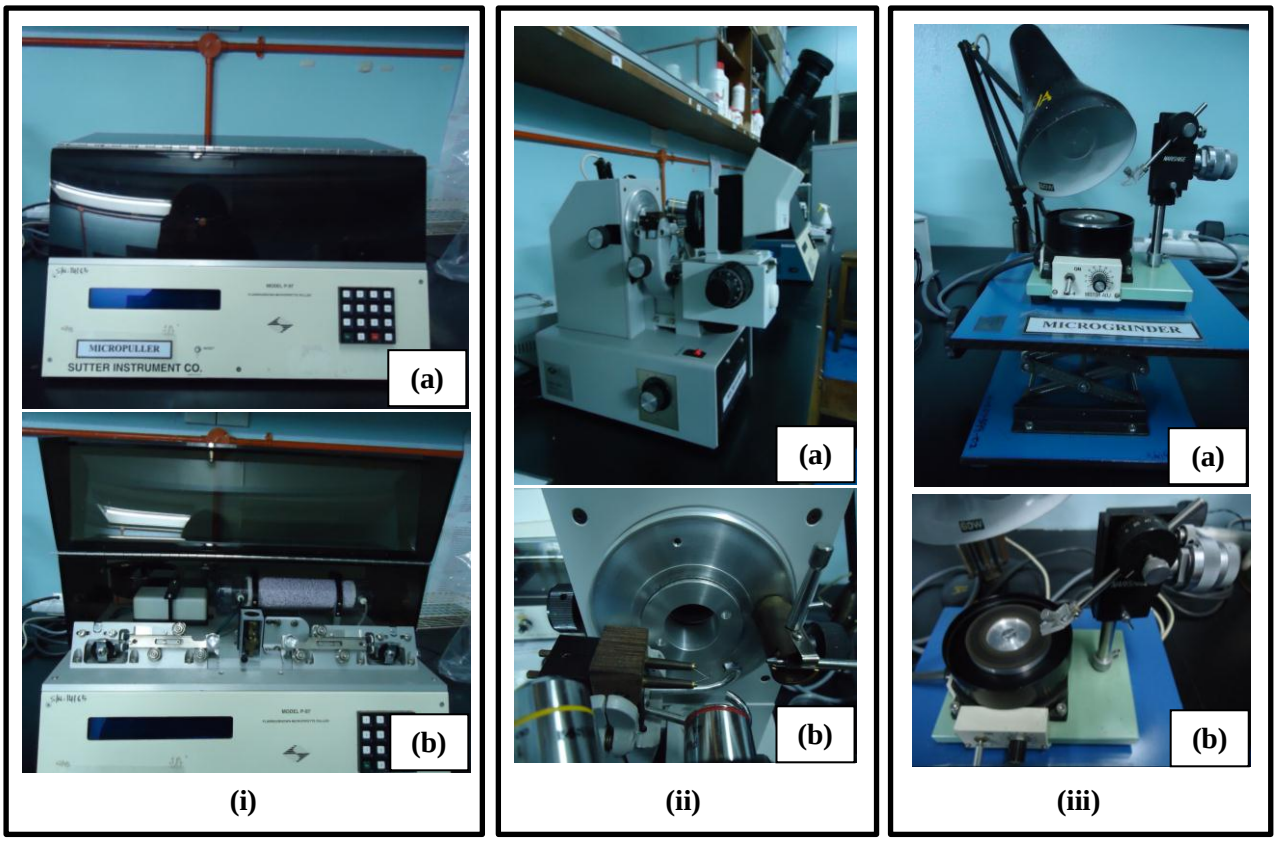


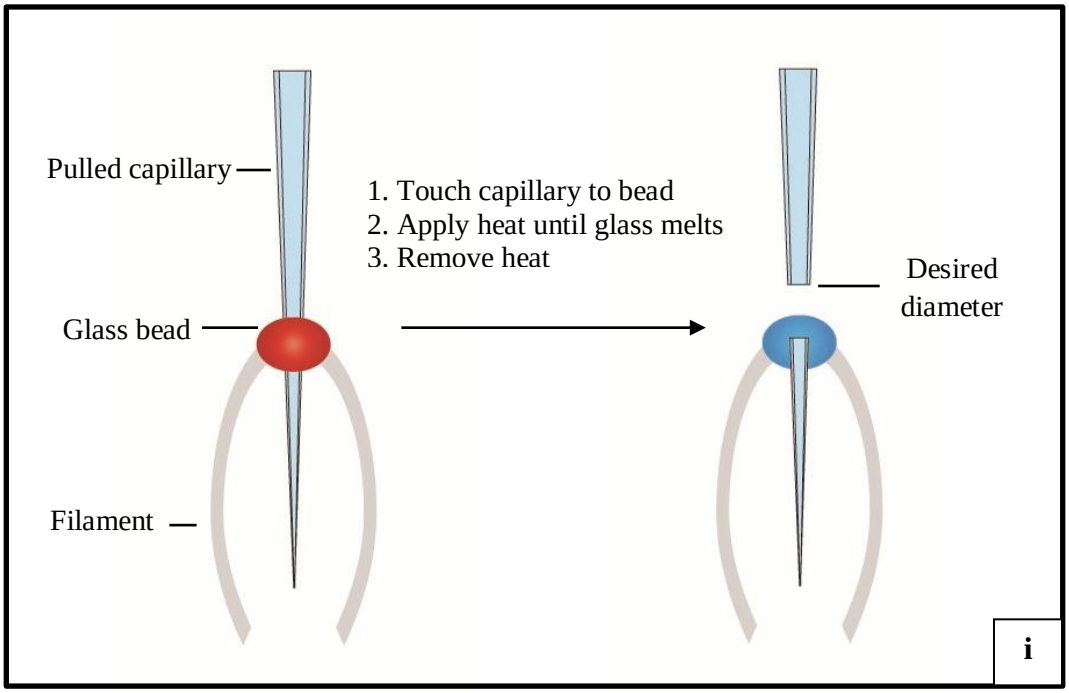
Figure 3.3: Equipment for microtools preparation. (i) Micropipette puller: (a) front-view, (b) mechanical elements; (ii) Microforge: (a) side-view, (b) closed-up view of microtools fabricating site; (iii) Microgrinder: (a) front-view, (b) closed-up view of microtools grinding site.

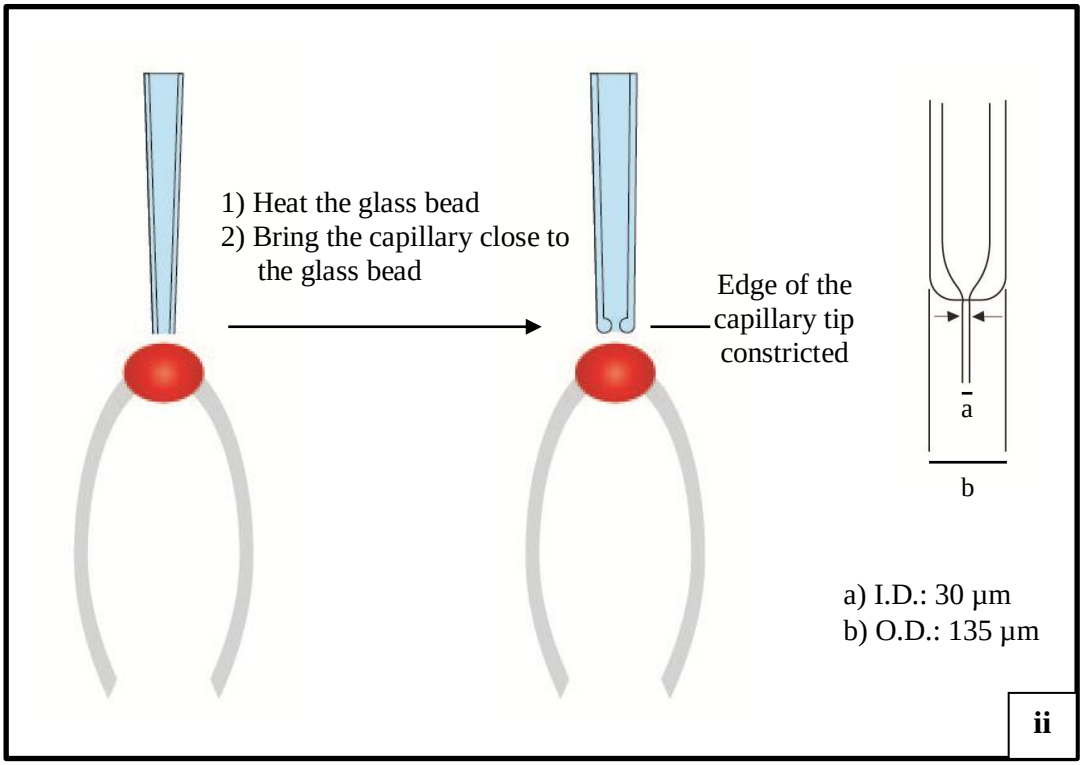
3.3.4.1 Cleaning and sterilisation of capillaries

The borosilicate glass capillaries were washed thoroughly with RO water. Subsequently, the borosilicate glass capillaries were soaked overnight in hydrochloric acid solution (10%). After 24 hours, the borosilicate glass capillaries were rinsed vigorously with Milli-Q water approximately twenty times to ensure all the traces of the acid completely removed. The borosilicate glass capillaries were kept in a conical tube (50 ml) and sterilised by autoclaving it. The sterilised capillaries were dried in the oven at 56°C, overnight.

3.3.4.2 Preparation of holding pipette

The holding pipette was used to hold and position oocytes for enucleation and sub-zonal injection of the donor cell in this study. The holding pipette with the inner diameter (I.D.) of 25 to 30 µm and outer diameter (O.D.) of 120 to 130 µm were fabricated using the microforge. In brief, the pulled capillary and filament were aligned to the same focus point. Then, the pulled capillary was placed on the glass bead at its required breaking point. The filament was heated to about 40 to 50% of its maximum capacity. As soon as the capillary melts to the glass bead, the power to the heat control was switched off. As the glass cools, the capillary will stick to the glass bead and once the filament relaxes from its expansion, the capillary was eventually broken evenly (Figure 3.4 (i)). The unwanted portion of the capillary that stick to the glass bead was removed. The glass bead was again heated up at about 60% of the maximum capacity and the tip of the capillary was moved closer to the glass bead so that the glass will melt and the inside diameter will shrink (polishing the tip) (Figure 3.4 (ii)). The micrometer scale in the eyepiece was used to judge when to stop the polishing process. The holding pipette was then bent at 30 to 35° angle using the heated glass bead (heat level= 50% of the maximum capacity) to allow a horizontal displacement on the microscope stage.





Note: (I.D.: inner diameter, O.D.: outer diameter)

Figure 3.4: Preparation of holding pipette. (i) Cutting of the pulled capillary; (ii) Fire-polishing of the capillary tip.

3.3.4.3 Preparation of enucleation needle

The enucleation needle was used in the process of removing the genomic DNA of the matured oocyte via squeezing approach. The sharp end needle was used to pierce a hole through the zona pellucida above the first polar body (PBI). Subsequently, the polar body and 10% of the cytoplasm adjacent to it was squeezed out through the breaking point of the zona pellucida. The sharp end of the enucleation needle was shaped using microforge. The pulled capillary was placed vertically with its tip touching the heated glass bead. Once the glass tip started to melt, the capillary was pulled up and down until a sharp needle end shape was formed (Figure 3.5 (i)). The enucleation needle was ultimately bent at 30 to 35° angle using the heated glass bead (heat level= 50% of the maximum capacity) to allow a horizontal displacement on the microscope stage (Figure 3.5 (ii)).

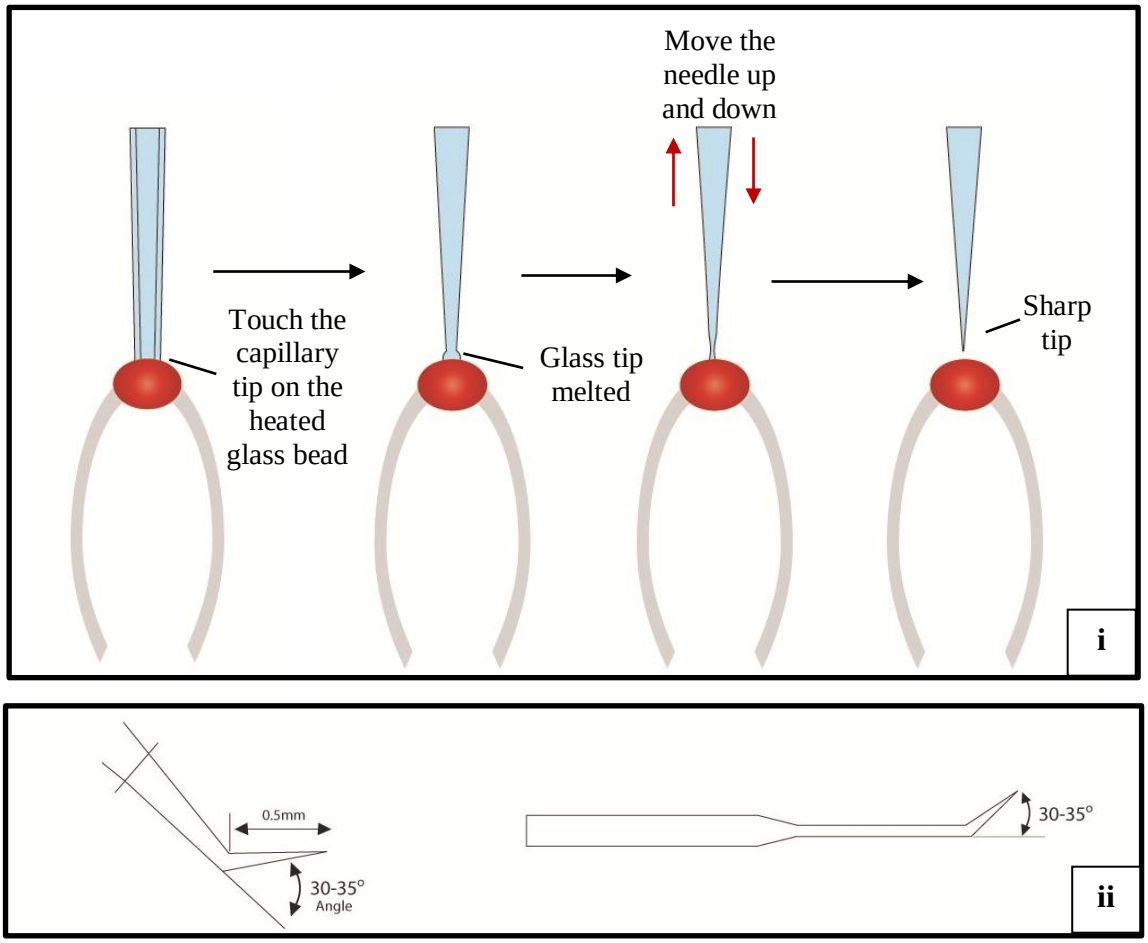
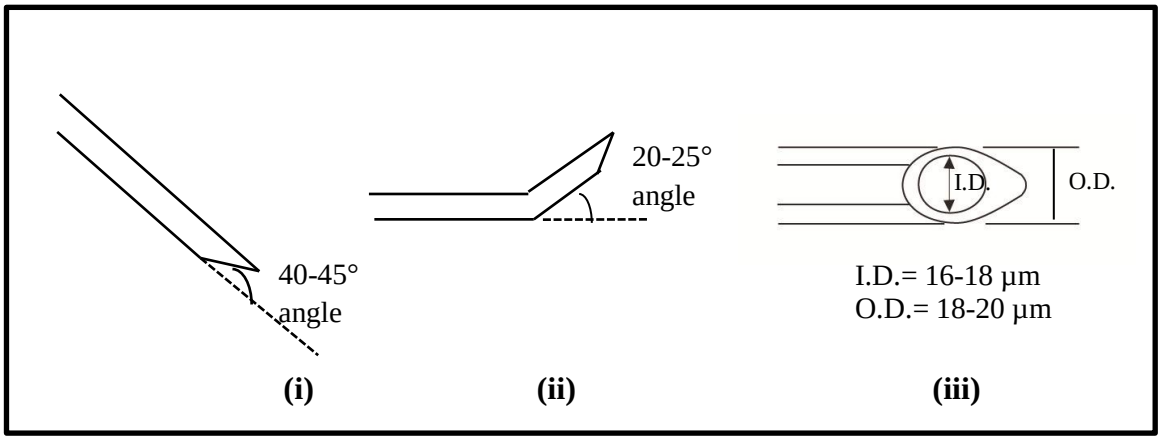


Figure 3.5: Preparation of enucleation needle. (i) Procedure to shape a sharp tip; (ii) Tip of the enucleation needle showing measurement of tip and angle of bending.

3.3.4.4 Preparation of injection pipette

The injection pipette was used to introduce a single donor karyoplast into the perivitelline space of the enucleated oocyte. The injection pipette was fabricated by cutting the tip of the capillary to the desired diameter (I.D.=16 to 18 µm; O.D.= 18 to 20 µm) using the microforge (Figure 3.4 (i)). Subsequently, the tip of the capillary was grinded approximately 3 to 4 minutes using a microgrinder to produce a bevelled edge with an angle of 40-45° (Figure 3.6). The capillary was then clean with EtOH (7%) and followed with Milli-Q water to remove the glass fragments accumulated on the grinded tip using an injection unit that assembled using an airtight syringe connected to the micropipette holder by a Teflon plastic tubing. The capillary was ultimately bent at 20 to 25° angle using the heated glass bead (heat level= 30% of the maximum capacity) to allow a horizontal displacement on the microscope stage.



Note: (I.D.: inner diameter, O.D.: outer diameter)

Figure 3.6: Preparation of injection needle. (i) Bevelled tip with the angle unit; (ii) Angle of bending; (iii) Measurement of the inner diameter and outer diameter.

3.3.5 Preparation of Caprine Donor Cell (Donor Karyoplast) Culture

Caprine (Jermasia goat) ear skin fibroblast cells were used as the donor karyoplast in the production of caprine intraspSCNT and interspSCNT embryos. Prior to ear tissue biopsy, the Jermasia goat was anesthetised via intramuscular (i.m.) injection (1 ml) of

mixed Xylazine hydrochloride and Ketamine hydrochloride (1:50). Then the surface of ear tissue to be biopsied was shaved to remove the hair. The incision area was swabbed with EtOH (7%) and by using a pair of surgical scissor, a piece of the ear tissue (~15-20 mm^2) was biopsied. The wounded area was then swabbed with EtOH (7%) and sprayed with iodine. The biopsied ear tissue was rinsed with mDPBS (3 times) before transported to the laboratory at 4°C in mDPBS.

In the laboratory, the biopsied ear tissues were rinsed again (3 times) with mDPBS before removing any remaining hair on the ear tissue by using a blade. The ear tissue was sprayed with EtOH (7%) and washed in primary cell culture medium (αMEM + 10% FBS + 3x penicillin-streptomycin) (3 times) before dissecting apart the dermal layer from the cartilage. The dissection was conducted using a blade clamped with a pair of haemostat. Subsequently, the dermal layer was minced (~3-5 mm^2) with a pair of sterile surgical scissor. The minced dermal tissues were then arranged at the centre of the tissue culture dish (60 mm, diameter) with the white surface facing down touching the bottom of the dish. In order to avoid the minced dermal tissues from floating, a glass cover slip was placed on top of the tissues. The primary tissue culture medium was then slowly dispensed into the tissue culture dish until the glass cover slip was fully immersed (~5 ml). This explant culture was incubated under a humidified atmosphere of 5% CO_2 in air at 37°C for 8 to 10 days. Once the outgrowth of cells was observed, usually from day 5 onwards, the primary explant culture medium was replenished with the cell line tissue culture medium that contain lower concentration of penicillin-streptomycin [1x]. The procedures of the explant culture were shown in Figure 3.7.

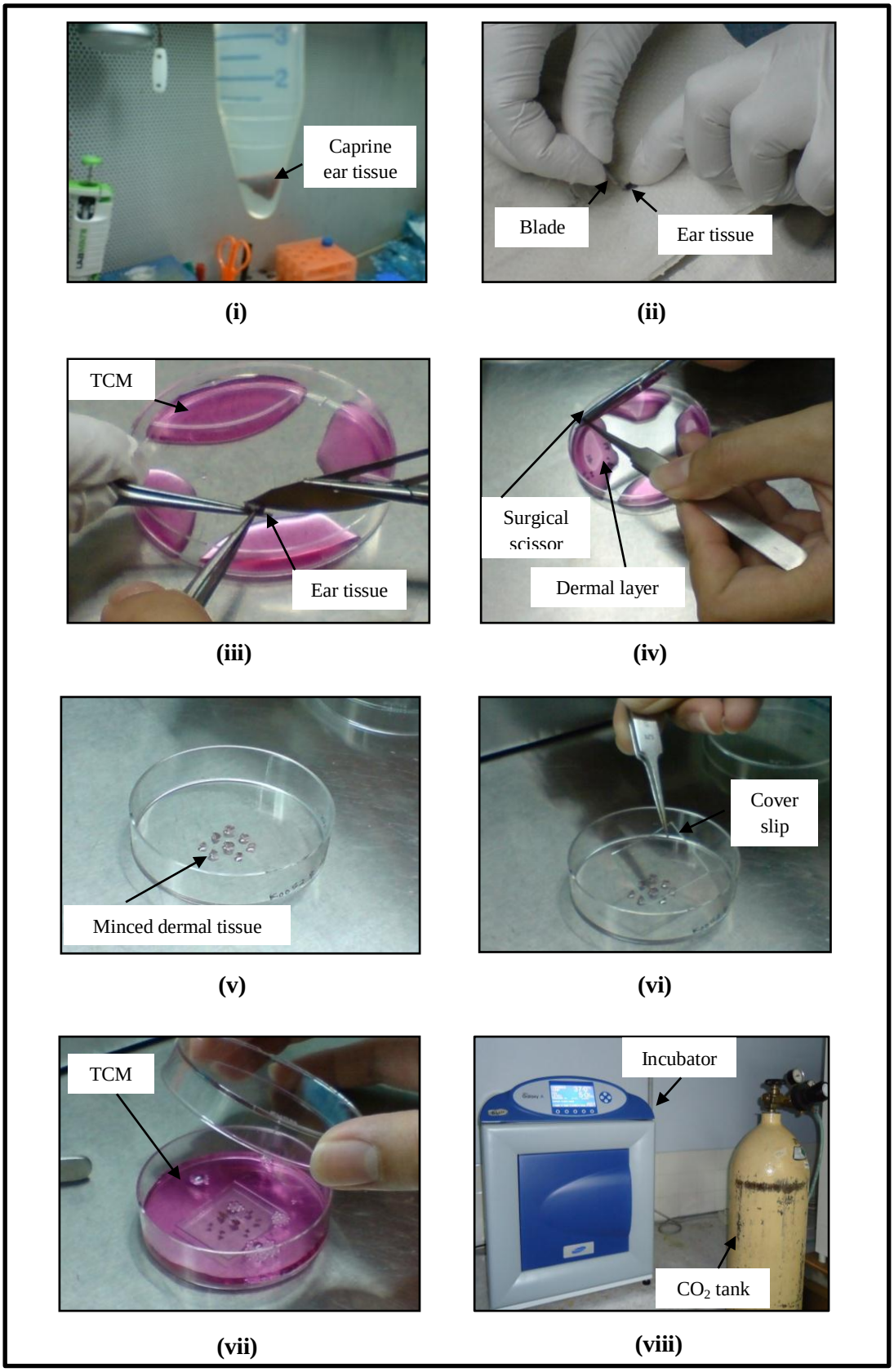


Figure 3.7: Procedure of caprine explant culture (ear tissue). (i) Caprine ear tissue biopsied; (ii) Removal of hair; (iii) Dermal layer dissection; (iv) Dermal layer mincing; (v) Arrangement of minced dermal tissue; (vi) Placement of glass cover slip; (vii) Tissue culture medium dispensed; (viii) Incubator for tissue culture.

At sub-confluence, outgrowths of the ear fibroblast cells were harvested using trypsin-EDTA solution. The harvested cells in the trypsin-EDTA solution were washed in the tissue culture medium by centrifuging it for 5 minutes at 1.5 rpm. The ear fibroblast cells in pellet form were suspended in the cell line culture medium and seeded in a culture flask (25 cm^2). The ear fibroblast cells were cryopreserved at passage 2 to 3 in freezing medium (cell line culture medium + 10% DMSO) and kept in liquid nitrogen. The frozen ear fibroblast cells were thawed and cultured in culture dishes (35 mm diameter) at least 3 days in advance before conducting nuclear transfer. A day before the nuclear transfer commence, serum starvation was done to synchronised cell cycle of the caprine ear fibroblast cell to be in G0 phase.

3.3.6 Preparation of Caprine and Bovine Recipient Cytoplast Culture

In this study, caprine oocytes were retrieved using two methods namely, LOPU and slicing of abattoir-derived ovaries, while bovine oocytes were retrieved solely from abattoir derived ovaries.

3.3.6.1 Caprine oocyte retrieval through LOPU procedure

Laparoscopic Oocyte Pick-up (LOPU) is a microsurgical procedure to retrieve oocytes from matured follicles in the ovary via four incisions on the abdomen of the does. Prior to LOPU, the donor does were oestrus synchronised and superstimulated. This procedure was carried out in Experiment 1, 2, 4 and 5.

3.3.6.1(a) Caprine hormonal stimulation protocol

In Experiment 1, a comparative study between two hormonal stimulation protocols was conducted. The schematic representation of the two hormonal stimulation protocols was shown in Figure 3.8.

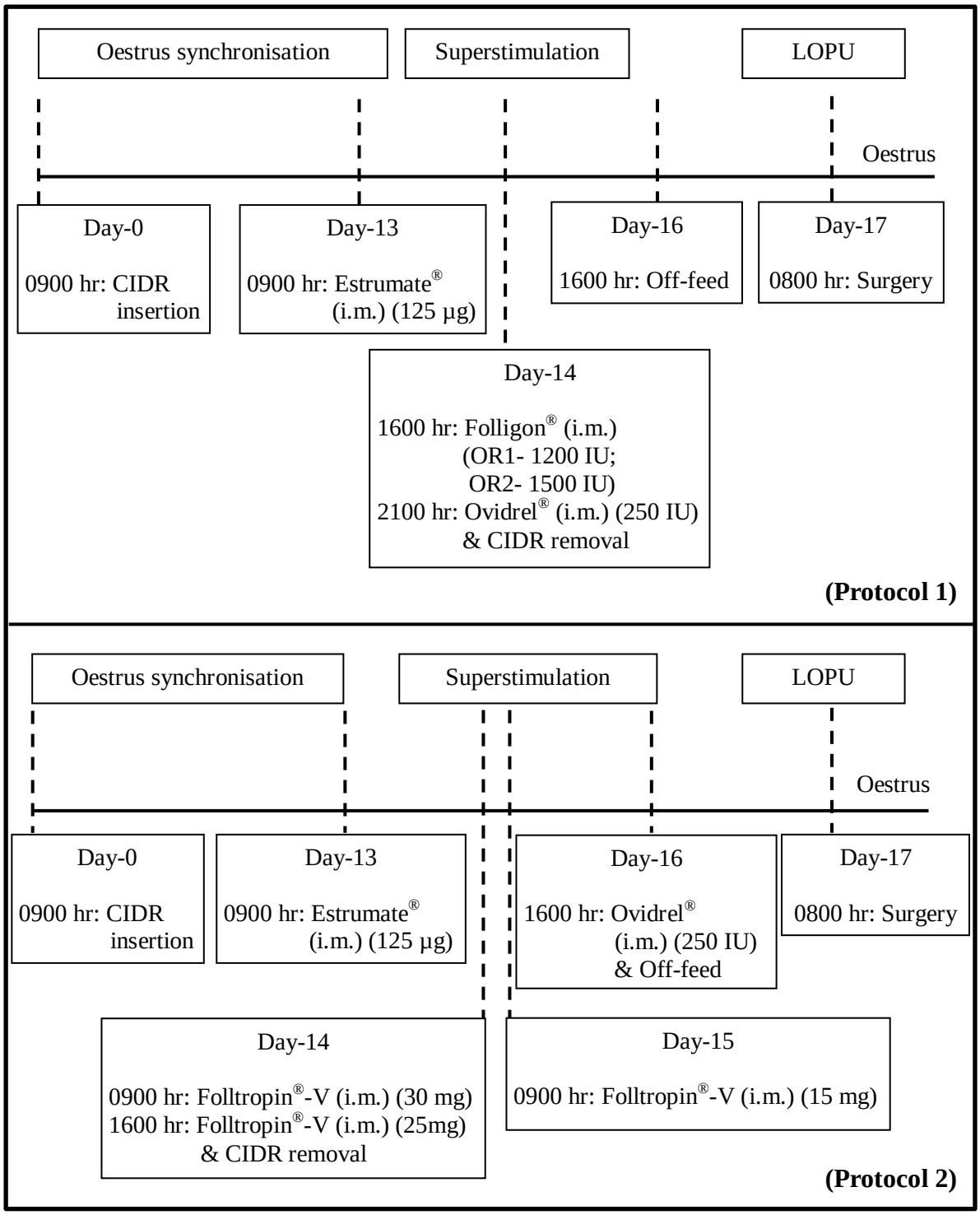


Figure 3.8: Schematic representation of caprine hormonal stimulation protocols. (Protocol 1) Hormonal stimulation using Folligon®; (Protocol 2) Hormonal stimulation using Folltropin®-V.

3.3.6.1(b) Oestrus synchronisation of donor does

In order to plan the date and time of oocyte retrieval (OR) via LOPU, oestrus synchronisation was conducted to manipulate the oestrous cycle of the donor does. The oestrous cycle was synchronised by the insertion of a controlled internal drug release device (CIDR®, 0.3 g progesterone) into the vagina of the donor doe for 14 days (at 0900 hour on Day-0) before removal. CIDR is made of an inert silicone elastomer that is non-porous and does not readily absorb bodily fluids; once it was inserted deep into the vagina with the help of a sterile CIDR applicator coated with lubricant (K-Y Jelly), the CIDR unfolds into a 'T' like formation that aids in retention. Daily monitoring of the device was performed to ensure that it had not been inadvertently removed. At approximately 36 hours prior to CIDR removal, a luteolytic treatment of Cloprostenol (Estrumate®, 125 µg) was administered intramuscularly (i.m.) (at 0900 hour on Day-13) to regress corpus luteum that facilitates initiation of pro-oestrus and eventually resulted in oestrogen sure for a rapid and highly visible onset of heat (oestrus).

3.3.6.1(c) Superstimulation of donor does

Superstimulation is a procedure to stimulate the growth of multiple follicles in the ovary. This procedure was conducted following oestrus synchronisation by the administration of hormonal treatments. The effect of superstimulation is morphologically seen as several fluid-filled 'pimple-like' protrusions on the surface of the ovary (Figure 3.12). Upon removal of CIDR, the donor does were superstimulated by administering FSH (Folligon® or Folltropin®-V) and hCG (Ovidrel®) at doses and intervals depending on the protocols designed (Figure 3.8). Protocol 1 was designated using PMSG, Folligon® (1200 IU on does in oocyte retrieval cycle 1 (OR1) while 1500 IU was used on does in OR2) and hCG, Ovidrel® (250 IU); while Protocol 2 was designated using pFSH,

Folltropin®-V with a total equivalent to 70 mg/doe, administered in decreasing doses (twice at Day-14 and once at Day-15) and hCG, Ovidrel® (250 IU). In Experiment 1, the superstimulation effect of using Protocol 1 vs. Protocol 2 was studied. As for Experiment 2, 4 and 5, Protocol 2 was used. At approximately 48 hours after PMSG or pFSH and hCG administration for Protocol 1 and 2, the donor does were observed for the onset of oestrus behavior such as switching of the tail, increased vaginal secretion and willingness to be mounted on by a teaser buck. The onset of oestrus marked the urgency of initiating LOPU procedure.

3.3.6.1(d) Anaesthesia and sedation of donor does

The donor does were deprived of food and water for 16 hours (at 1600 hr on Day-16) prior to LOPU. On the day of performing LOPU, anaesthesia was induced with intramuscular administration of mixed Xylazine hydrochloride and Ketamine hydrochloride (1:50) at the dosage of 1 ml per 25 to 30 kg body weight. The doe was maintained under anaesthesia with the dosage of 0.5 ml every 20 to 30 minutes interval or as required.

3.3.6.1(e) Disinfection of surgical instruments and skin area of doe

On the day prior to surgery, non-autoclavable surgical instruments such as atraumatic grasper, trocar and cannula, fibre optic cable, light probe for endoscope, silicone tubing of flushing system and gas tubing with luer lock at both ends for CO_2 system were disinfected by immersing completely in Gigasept® solution (10%) for 10 minutes and subsequently rinsed with sterile autoclaved distilled water before placing on a sterile surgical table-cum-trolley. Autoclavable surgical instruments were assembled and

arranged in a consistent order on a sterile table-cum-trolley which was already covered with a sterile drape (Figure 3.9).

When the donor doe had been immobilised, it was fasten on a restraining cradle at dorsal recumbence tilted at approximately 45° angle. The abdominal area of the doe was scrubbed with diluted Hibiscrub (10%) using gauze for disinfection purpose. Subsequently, the hair was shaved and the bare skin was wiped with undiluted Hibiscrub before applying the surgical iodine solution on the surgical surface. A sterile drape (tailor-made cloth with an opening that revealed the disinfected bare skin) was then used to cover the abdominal and its position was secured in place using towel clamps.

3.3.6.1(f) Laparoscopic ovum pick-up (LOPU)

The surgical instruments (Figure 3.9), laparoscopic system and accessories used were purchased from Aesculap A.G. & Company, Germany (Appendix Table 1.1). The condition of the laparoscopic system and accessories were inspected regularly (weekly basis) to ensure no disruption occur due to machine break down during LOPU.

Typically, the LOPU procedure was conducted by the surgeons (Figure 3.10). The procedure began by assembling the light probe (a paediatric Storz laparoscope connected to a light system via a fibre optic cable). Subsequently, the light probe was connected to the Aesculap endoscopic camera which was connected to the CCD camera and monitor beforehand. The lens of the paediatric Storz laparoscope, fibre optic cable and the endoscopic camera system were wiped with EtOH (7%) soaked sterile gauze. The CCD camera was switched on, video system was tested and a white balance was performed. Subsequently, the ovum pick-up (OPU) needle, test tube (14 ml) and syringe

containing flushing medium (50 ml) were connected to the flushing and aspiration system prior to introducing any incision on the doe.

A pneumoperitoneum was created by inserting CO_2 gas into the abdominal of the doe through the Verrus needle attached to the plastic tubing that was connected to a CO_2 tank via CO_2 gas insufflators unit. Once the pneumoperitoneum was created, small incision (3-5 mm) were made, one near the umbilicus to facilitate insertion of trocar for passing the laparoscope, one on the right side of lower-ventral abdomen to insert the trocar for passing aspiration needle. The paediatric Storz laparoscope that was connected to the CCD camera was inserted into the abdominal cavity through the trocar sheath. Then a paediatric grasper was passed through the small trocar sheath and finally the OPU needle was inserted into the left side of the abdominal cavity. The ovaries were visualised with the help of laparoscope and exposed by pulling the fimbria in different directions with the grasper. The number of visible antral follicles (2-3 mm sized or larger) on both ovaries were counted and recorded. Subsequently, one of the ovaries was held by the grasper and follicles were individually punctured, flushed with flushing medium and then the flushing medium plus the oocytes were aspirated back into the OPU needle to be collected into the test tubes (2-4 ml per tube). The collection tubes were then passed to the researcher for oocyte search. The procedure was repeated for the other ovary. The images of LOPU procedure and superstimulated ovary were shown in Figure 3.10- 3.12.

Figure 3.9: Surgical instruments and accessories.

Figure 3.10: Surgeons conducting LOPU procedure.

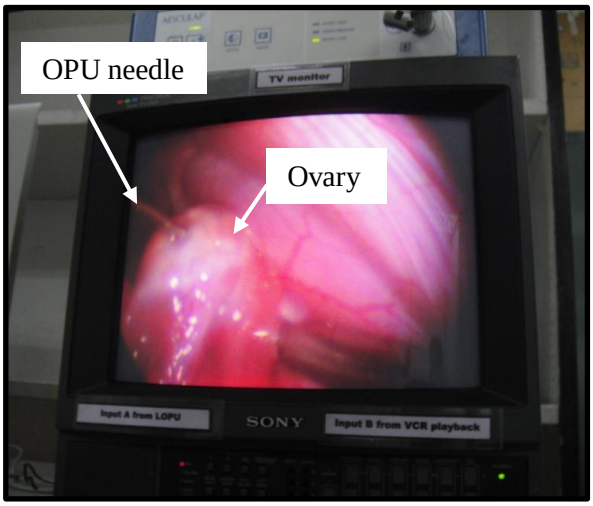


Figure 3.11: Closed up image of the ovary during LOPU.

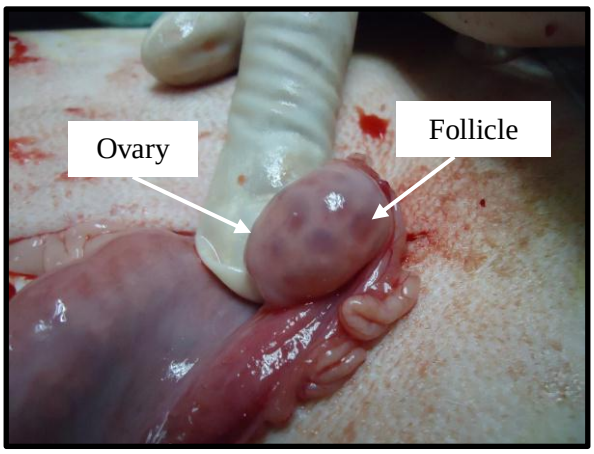


Figure 3.12: Superstimulated caprine ovary.

3.3.6.2 Oocyte retrieval from abattoir-derived ovaries

The abattoir-derived caprine and bovine ovaries were transported to the laboratory in ovary collection medium (normal saline supplemented with penicillin-G (60 µg/ml) and streptomycin (50 µg/ml)) at 30-37°C. All the ovaries were rinsed at least four times with ovary collection medium to remove off the blood prior oocyte retrieval. Oocytes were retrieved using two approaches namely ovary slicing and follicle aspiration (Figure 3.13). Typically, caprine oocytes were retrieved using only ovary slicing method due to the size of ovary and number of follicles that were relatively small compared to bovine.

In ovary slicing approach, the ovary was held with a sterile curved haemostat on the culture dish (60 mm diameter) and the excess tissues were cut with a sterile scissor. Subsequently, a quarter section of the stainless steel razor blade was held with a haemostat and checkerboard incision was made to the entire surface of the ovary by using the blade inside the culture dish containing pre-warmed (37°C) oocyte retrieval medium (4 ml). The sliced ovary was rinsed in a beaker (100 ml) containing oocyte retrieval medium (50 ml). All the steps in the process were repeated until all ovaries were sliced. Subsequently, the beaker and culture dish containing the oocyte retrieval medium and the sliced material were transfer into the embryo room for COCs pick-up under a stereomicroscope attached to a stage warmer (37°C). The beaker was left to stand (5 minutes) on a hot plate with the temperature maintained at 37°C in order to allow the tiny pieces of tissues and COCs in the beaker to settle down. While waiting for the sedimentation of the tissues and COCs to take place, COCs pick-up using the mouthpiece-controlled pipette from the culture dish was conducted. All the COCs collected were placed in a culture dish (35 mm diameter) containing oocyte retrieval medium (3 ml).

As for follicle aspiration approach, the ovary was held using a sterile tissue and by using a needle (18 Gauge) connected to a sterile syringe (10 ml), the follicles were punctured and the follicle fluid was aspirated into the syringe. The syringe was filled with oocyte retrieval medium before aspirating the follicle fluid. Once the COCs were completely retrieved from all the ovaries, the mixture of oocyte retrieval medium and follicle fluid was dispensed from the syringe into the conical tube (50 ml) containing fresh oocyte retrieval medium (20 ml). The conical tube was left to stand on a rack (5 minutes) in order to allow the COCs to sediment. Some of the sediment (1 ml) were transferred into a culture dish (60 mm diameter) and diluted with oocyte retrieval medium (2 ml) to ease the observation and pick-up of COCs under the stereomicroscope attached to a stage warmer (37°C). All the COCs collected were placed in a culture dish (35 mm diameter) containing oocyte retrieval medium (3 ml) prior to grading and *in vitro* maturation (IVM).

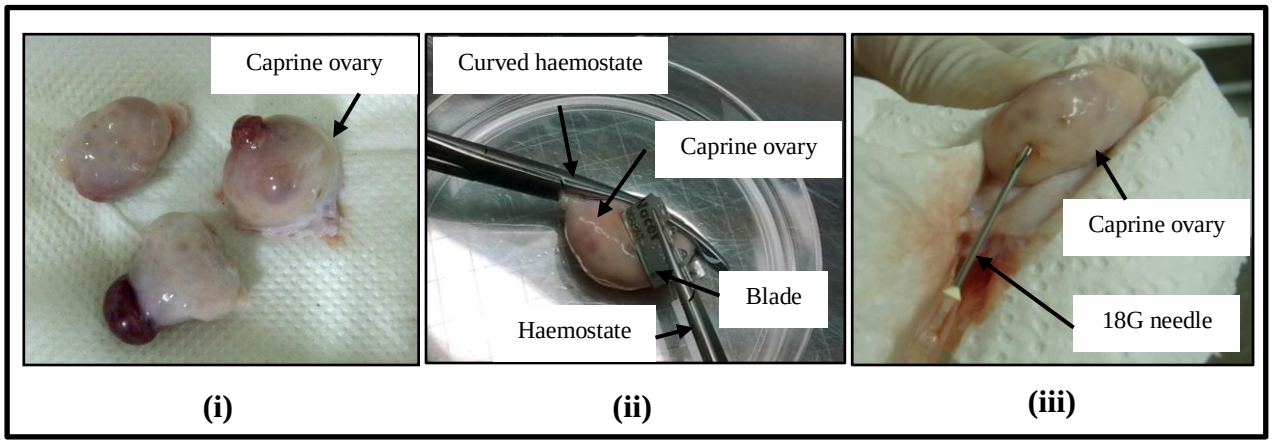


Figure 3.13: Oocyte retrieval from abattoir-derived ovaries. (i) Caprine ovaries; (ii) Caprine oocyte retrieval via slicing technique; (iii) Bovine oocyte retrieval via aspiration technique.

3.3.6.3 Oocyte grading

All the COCs collected via LOPU and abattoir-derived ovaries were washed in oocyte retrieval medium (3 times) before visually assessed under the stereomicroscope to classify in grades according to cumulus cell investment and morphology of the oocyte. The classification of the COCs in this study was based on the criteria described by Rahman *et al*. (2008), presented in Table 3.38.

Table 3.38: Grading of the recovered oocytes according to the cumulus cell investment and morphology of the oocyte

Grades	Characteristics of COCs and CFOs
A	COCs with more than 5 complete layers of cumulus cells (CCs), finely granulated homogeneous ooplasm and normal morphological features.
B	COCs with 3-5 complete layers of CCs, finely granulated homogeneous ooplasm and normal morphological features.
C	COCs with 1-2 complete layers of CCs or COCs with 3-5 partially invested CC layers, finely granulated homogeneous ooplasm and normal morphological features.
D	CFOs or oocyte with incomplete investment of CCs (1-2 layers), finely granulated homogeneous ooplasm and normal morphological features.
E	Degenerating oocyte or oocyte with abnormal size, shape and heterogeneous ooplasm or apoptotic oocytes in jelly-like CC investment or very small oocytes.

Adapted from: Rahman *et al*. (2008)
Note: COCs – Cumulus oocyte complexes; CFOs – Cumulus free oocytes.

3.3.6.4 *In vitro* maturation (IVM)

In vitro maturation medium (5 ml) was equilibrated in the incubator (5% CO_2 in air in humidified atmosphere, 38.5°C) overnight (or 12 hours). Microdroplets (70 µl) of IVM medium were prepared and overlaid with equilibrated light mineral oil in a small polystyrene culture dish (35 mm diameter) on the day of experiment. The extra IVM medium (~3 ml) was kept in the incubator for COCs washing after grading before culturing in the IVM microdroplets. The COCs were cultured according to grade in different microdroplets (13-15 COCs/ 70 µl) of IVM medium under a humidified atmosphere of 5% CO_2 in air, 38.5°C. The COCs collected in this study were cultured in IVM medium according to the following duration:

a) Caprine COCs retrieved from LOPU: 18-22 hours or 23-27 hours of IVM duration (A comparative study in Experiment 4)
b) Caprine COCs retrieved from abattoir derived ovary: 22-26 hours of IVM duration
c) Bovine COCs retrieved from abattoir derived ovary: 22-24 hours of IVM duration

3.3.6.5 Assessment of oocyte maturation

In Experiment 2, assessment of the oocyte stages at 3 hours of IVM intervals (from 15-27 hours after COCs cultured in IVM) were conducted by staining the denuded COCs with Hoechst 33342. On the other hand, assessment of the matured oocytes for Experiment 3, 4 and 5 were conducted at the IVM duration mentioned in section 3.3.6.4. Once the IVM duration approached, the COCs were treated with hyaluronidase (0.1%) not exceeding 5 minutes. The cumulus cells were stripped off the zona pellucida by

repeated pipetting (40-50 times) using micropipette (200 µl) adjusted to 100 µl in the hyaluronidase (0.1%) solution. Subsequently the denuded oocytes were washed in EMCARE^{TM} holding solution (3 times) to remove traces of hyaluronidase. The denuded oocytes were assessed for maturation under stereomicroscope by rotating the oocytes using mouthpiece-controlled pipette. Oocytes with a clear protrusion of first polar body (PB-1) were considered as matured (in metaphase II) and meiotic competent. The matured oocytes were kept in incubated oocyte holding solution for subsequent nuclear transfer manipulation and parthenogenesis treatment (Experiments 3, 4 and 5). Oocytes without the present of PB-1 were considered immature and discarded.

3.3.7 Somatic Cell Nuclear Transfer Procedure

In this study, SCNT was carried out using 'Roslin technique' (a technique developed and used by Ian Wilmut from Roslin Institute, UK) that includes the following series of steps: (i) enucleation using squeezing technique, (ii) sub-zonal injection of donor karyoplast and (iii) electrofusion. All the three steps mentioned above were carried out using the micromanipulation system that incorporated an inverted microscope fitted with hydraulic micromanipulators and connected to a computer system (Figure 3.14). A summary on the SCNT procedure was shown in Figure 3.15.

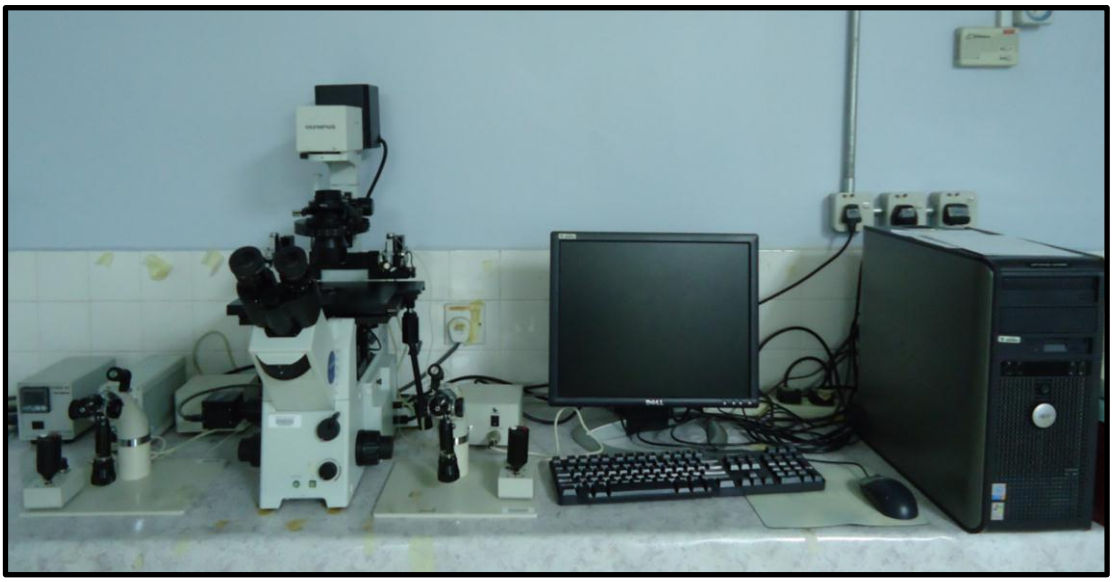


Figure 3.14: Micromanipulation system.

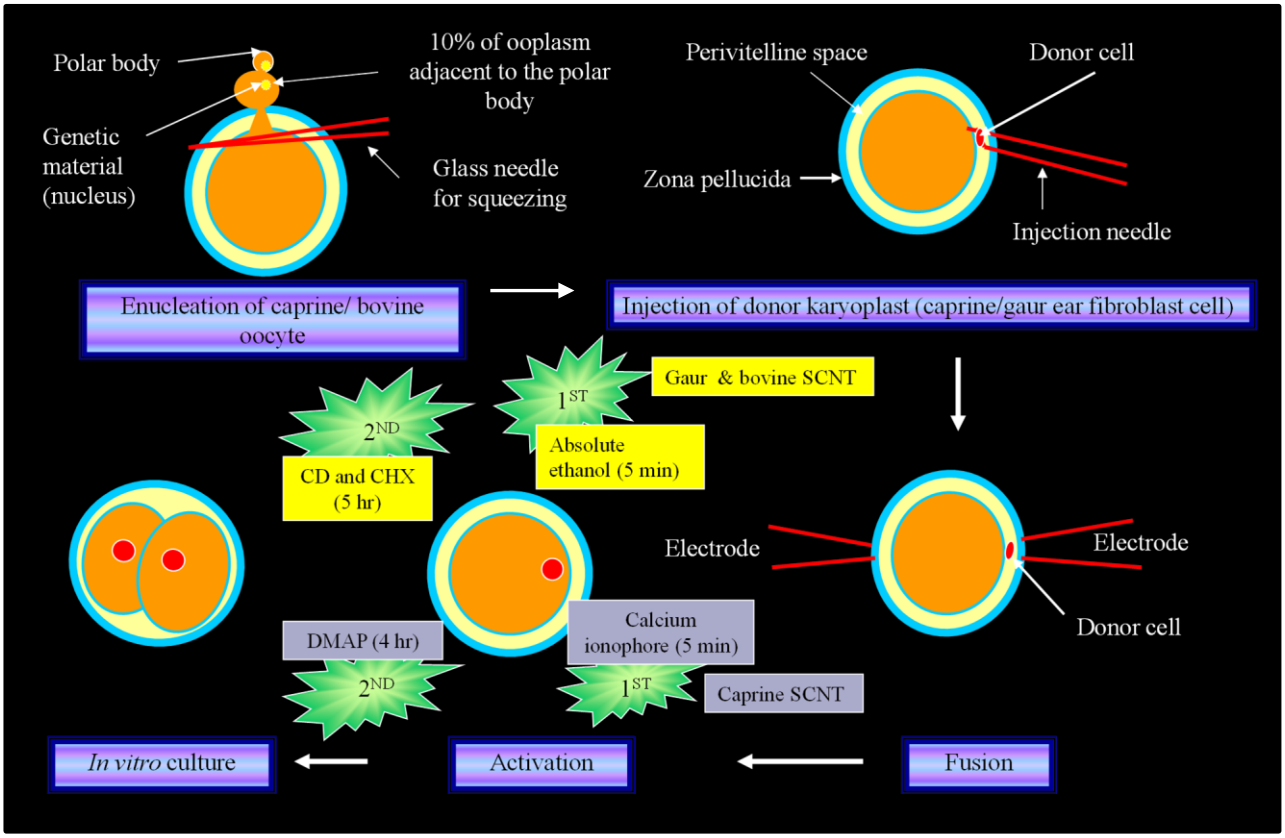


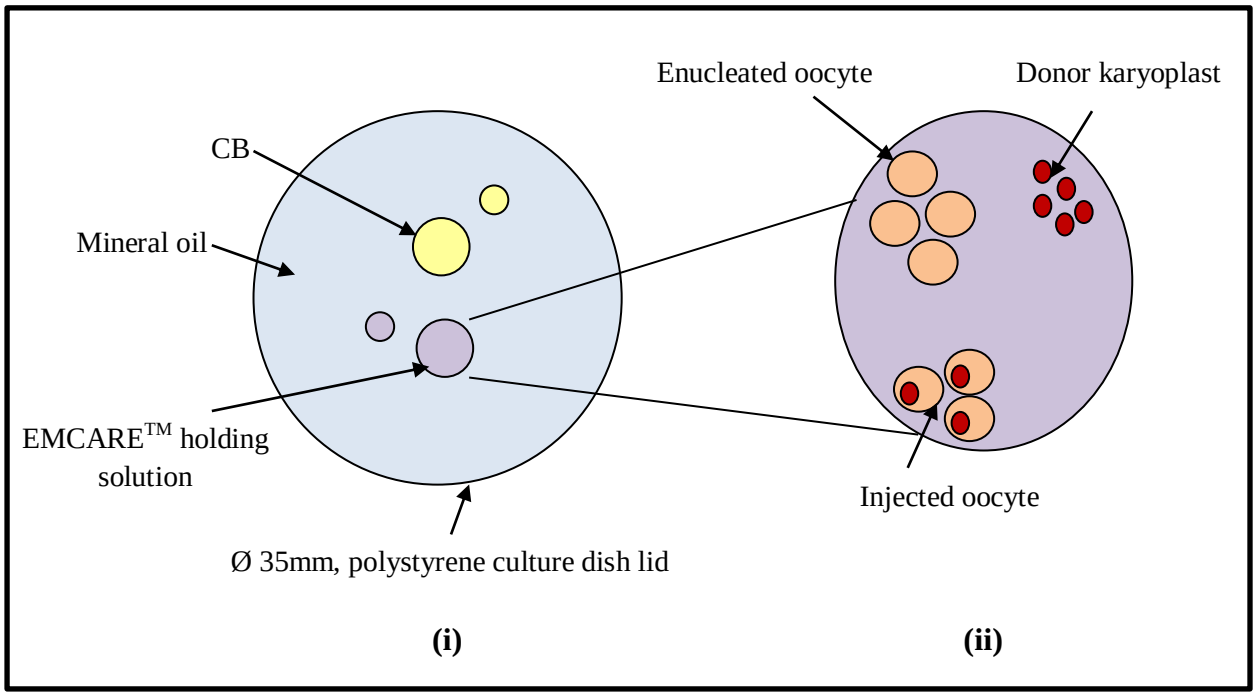
Figure 3.15: Summary of SCNT procedure.

3.3.7.1 Preparation of micromanipulation dish

Generally, three micromanipulation dishes (‘blank’ dish, nuclear transfer dish and electrofusion dish) were prepared on the day of SCNT experiment. The ‘blank’ dish was prepared for the alignment of the enucleation needle or injection pipette with the holding pipette. This dish was prepared by dispensing a microdroplet (30 µl) of EMCARE^{TM} holding solution onto the lid of a polystyrene culture dish (35 mm diameter) and covered with light mineral oil.

After the preparation of ‘blank’ dish, nuclear transfer dish was prepared by dispensing two microdroplets of CB solution (100 µl first: for enucleation purposes; 30 µl second: for washing the holding pipette and enucleation needle) on the lid of the polystyrene culture dish (35 mm diameter) at the upper end. Subsequently another two microdroplets of EMCARE^{TM} holding solution (100 µl; for donor karyoplast injection purpose and 30 µl; for washing the holding pipette and injection pipette) were dispensed on the opposite end of the dish (Figure 3.16).

The electrofusion dish was prepared for the fusion between the sub-zonal injected donor kayoplast with the recipient cytoplast using electrical pulse. The dish was prepared by drawing two parallel lines and another line inter-cross the centre point of the polystyrene culture dish (60 mm diameter) bottom with a labelling marker (Figure 3.17). Subsequently, 400 µl of Zimmerman’s fusion medium (ZFM), was dispensed at the centre of the marked polystyrene culture dish (60 mm diameter) and the microdroplet was covered with light mineral oil.



Note: (CB: cytochalasin B)

Figure 3.16: Nuclear transfer dish. (i) Arrangement of enucleation and donor karyoplast injection microdroplets on the nuclear transfer dish; (ii) Placement of samples in the microdroplet for donor karyoplast injection.

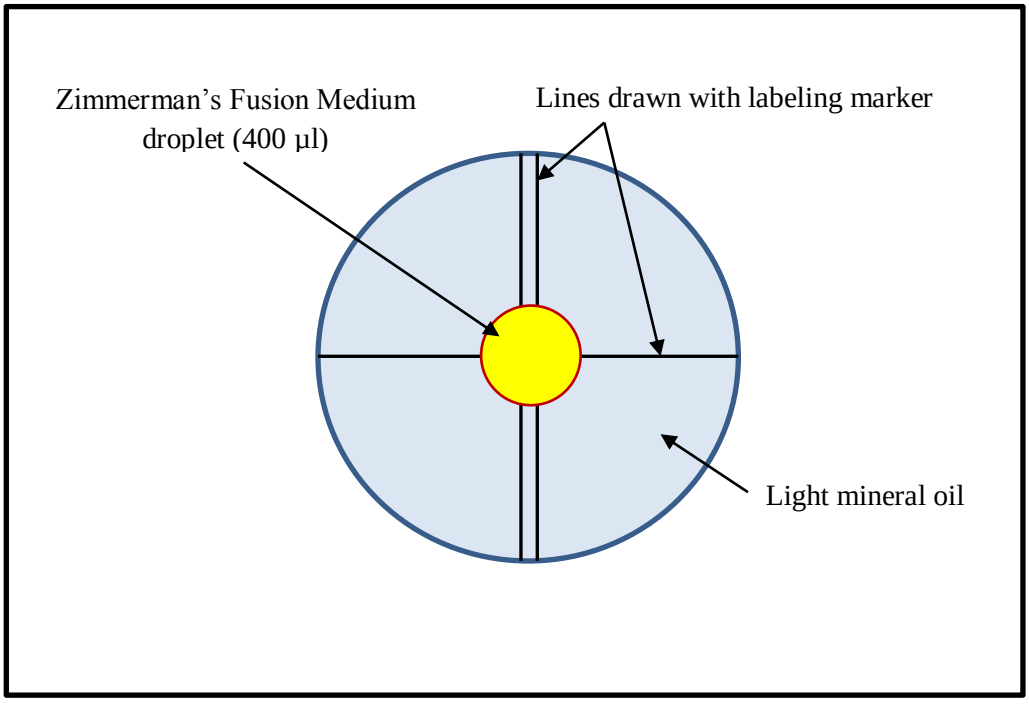


Figure 3.17: Electrofusion dish.

3.3.7.2 Alignment of microtools

Alignment of microtools is the preliminary and crucial step that needs to be carried out before the commencement of any micromanipulation procedure. The performance of the subsequent micromanipulation procedures is highly dependent on the accuracy of the microtools alignment. The microtools (holding pipette with enucleation needle or injection pipette) alignment was conducted by first adjusting all the knobs (X-, Y-, Z-control) and syringes (3 ml) to the centre of the scale.

The 'blank' dish prepared earlier was then placed on the centre of the heated stage warmer (38.5°C) of the micromanipulator. The microscope (under 4x objective) connected to the micromanipulator was adjusted until the edge of the alignment droplet was sharply focused. Subsequently, the holding pipette inserted to the injection holder was fixed to the universal joint located on the left side of the micromanipulator. On the other hand, the enucleation needle inserted to the electrode holder was fixed to the universal joint located on the right side of the micromanipulator. The position of the holding pipette was lowered down until the tip touched the oil. This position was held for a few minutes (1 to 2 minutes) to allow the light mineral oil flowed into the tip by capillary action. This step was eliminated for the enucleation needle but is required for the injection pipette. Once the tip was filled with light mineral oil, both holding pipette and enucleation needle were moved to the alignment droplet. The holding pipette was focused with the enucleation needle and was aligned so that the working tips were parallel to the microscope stage (under 4x objective) (Figure 3.18). Finally both microtools were checked under high magnification to assure the accurate alignment (sharply in focus) and parallel.

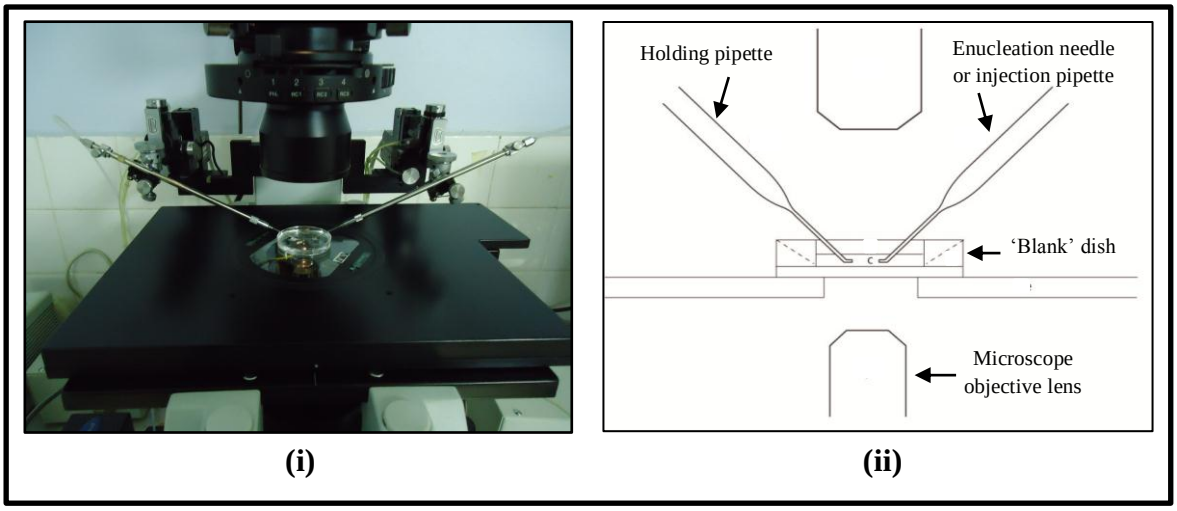


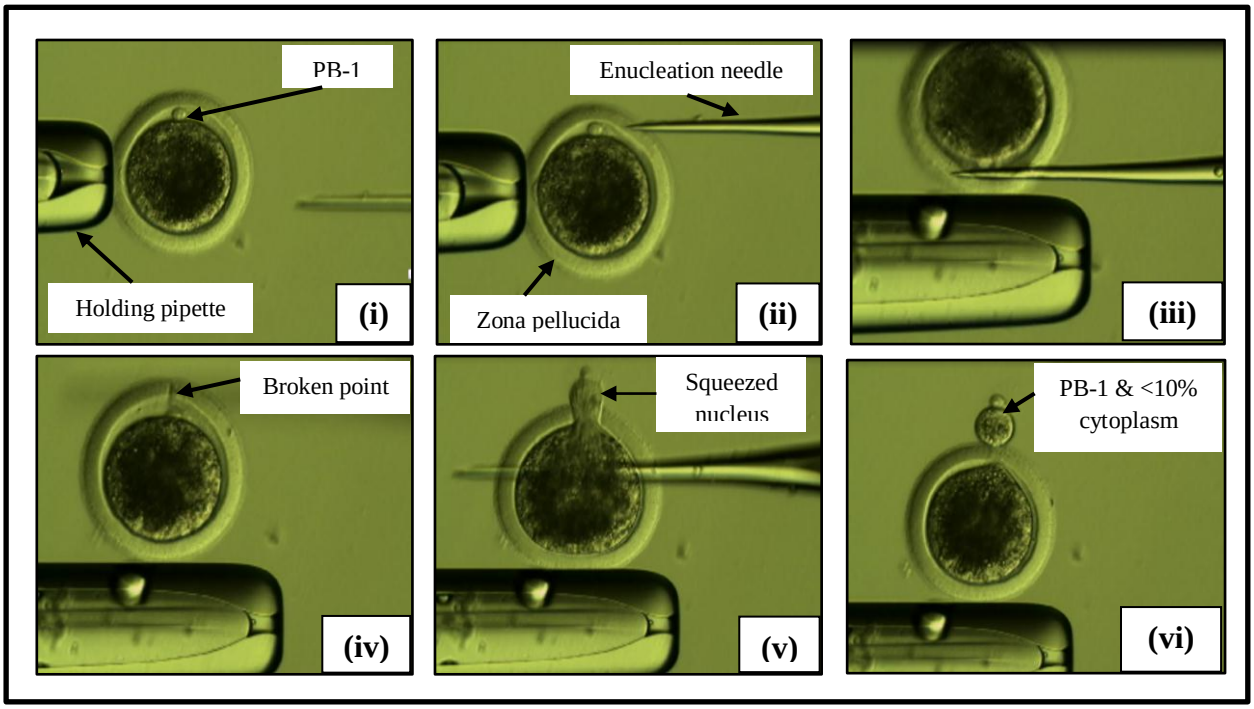
Figure 3.18: Alignment of microtools on the 'blank' dish. (i) Original photograph; (ii) Labelled photograph.

3.3.7.3 Enucleation of matured oocyte using squeezing method

In this study, squeezing technique was used to remove the genomic DNA from the matured oocyte. The matured oocytes were treated with cytochalasin B (5 µg/ml) solution for 5 minutes to destabilise the actin cytoskeleton before enucleation.

After the alignment of holding pipette and enucleation needle was completed, the 'blank' dish was replaced with nuclear transfer dish. The CB treated matured oocytes were then transferred into the CB droplet in the nuclear transfer dish using the mouthpiece-controlled pipette. By using the joysticks of the micromanipulator, the movement of the holding pipette and enucleation needle were controlled. The matured oocyte was rotated using the tip of the holding pipette and enucleation needle to locate the position of the first polar body. The oocyte was held firmly using the holding pipette with its first polar body (PB-1) located at 12 o'clock position. Subsequently, an enucleation needle was used to make a cut on the zona pellucida above the first polar

body. The first polar body and 10% of the cytoplasm adjacent to the polar body was gently squeezed out. In order to confirm that the oocyte was successfully enucleated, the extruded polar body and cytoplasm was stained with Hoechst 33342. Only oocytes with positive enucleation were subjected to subzonal injection of donor karyoplast. Exposure of the oocytes to CB solution for the entire enucleation step was ensured not to exceed 30 minutes. The enucleated oocytes were washed 3 times in EMCARE^{TM} holding solution and kept in the incubated holding medium (30-45 minutes) while waiting for the preparation of nuclear transfer via sub-zonal injection to complete. The micromanipulation procedure of oocyte enucleation using squeezing technique is shown in Figure 3.19.



Note: (photomicrographs original magnification: 100x); (PB-1: first polar body)

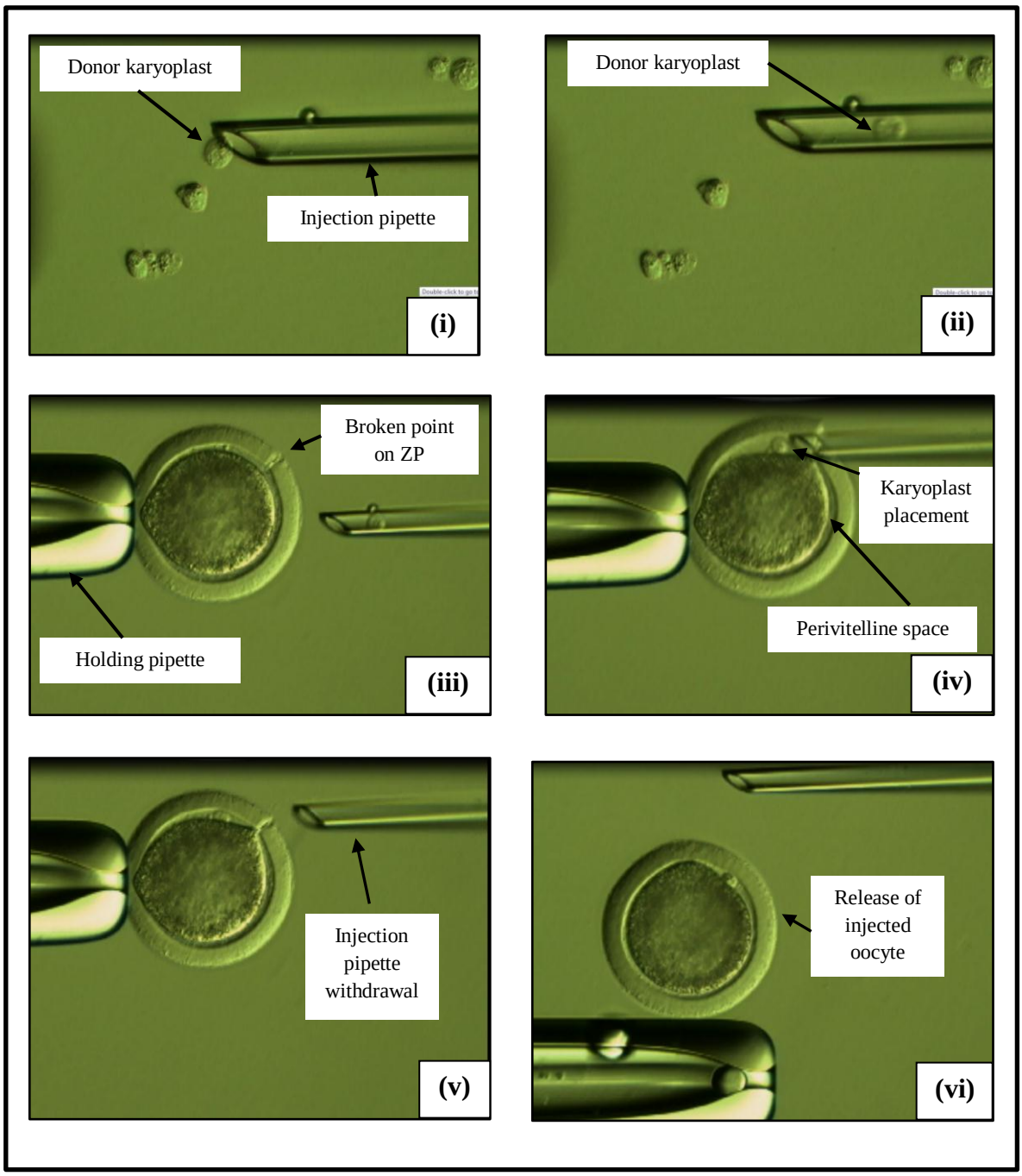
Figure 3.19: Oocyte enucleation using squeezing technique. (i) MII oocyte was held firmly with holding pipette with PB-1 located at 12 o'clock position; (ii) Enucleation needle was used to pierce through the zona pellucida above PB-1; (iii) The oocyte was released from the holding pipette and brought down to opposite side, few attritions were made between the enucleation needle and holding pipette to break the zona pellucida; (iv) Relocate the oocyte with the broken point at 12 o'clock position; (v- vi) The PB-1 and cytoplasm (<10%) beneath the PB-1 were gently squeezed out.

3.3.7.4 Nuclear transfer of the enucleated oocyte using sub-zonal injection method

Ear skin fibroblast cells (donor karyoplast) were harvested using the method described in section 3.3.5. The harvested cells in pellet form were suspended with EMCARE^{TM} holding solution (70-100 µl).

Once the preparation of donor karyoplast completed, the enucleation pipette was replaced with the injection pipette. The position of the nuclear transfer dish was adjusted in which the EMCARE^{TM} droplet (manipulation droplet) was in the centre of focus. Subsequently, the donor karyoplast suspension (10 µl) was dispensed into the manipulation droplet on the upper left corner, while the enucleated oocytes were transferred to the upper right corner (Figure 3.14 (ii)).

In order to inject the donor karyoplast into the perivitelline space of the enucleated oocyte, a single ear skin fibroblast cell was aspirated into the injection pipette and positioned near to its tip. By using the tip of the holding pipette and injection pipette, the enucleated oocyte was rotated to locate the oocyte in which its breaking point of the zona pellucida was at 1 to 2 o'clock. The oocyte was held firmly with the holding pipette and the tip of the injection pipette was inserted into the perivitelline space of the enucleated oocyte. Subsequently, the donor karyoplast was deposited into the perivitelline space at the point in which the donor cell could adhere closely to the recipient cytoplast. The injected oocytes were kept in the incubated holding medium for 30-45 minutes under a humidified atmosphere of 5% CO_2 in air, 38.5°C before fusing the couplets. The micromanipulation procedure of nuclear transfer using sub-zonal injection technique is shown in Figure 3.19.



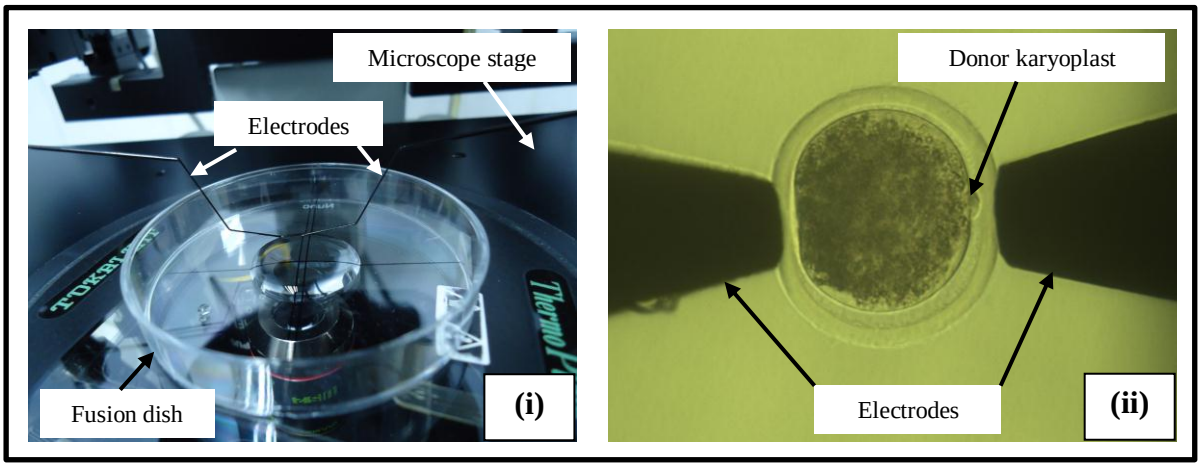
Note: (photomicrographs original magnification: 100x)

Figure 3.20: Nuclear transfer using sub-zonal injection technique. (i-ii) A single donor karyoplast cell was aspirated into the injection pipette; (iii) The oocyte was located in which the broken point of the zona pellucida was at 1-2 o'clock position; (iv-vi) The donor karyoplast was deposited into the perivitelline space of the enucleated oocyte through the breaking point of the zona pellucida.

3.3.7.5 Fusion of donor karyoplast and recipient cytoplast couplet

In this study, electrofusion method was used to fuse the donor cell- cytoplast couplets. Before introducing electrical stimulation, the couplets were equilibrated in fusion buffer (ZFM) formulated by Zimmermann and Vienken (1982) for 5 minutes.

The electrofusion was carried out using a fusion machine (SUT F-1, Suranaree University of Technology, Thailand) connected to a pair of chopstick type electrode. The electrodes were fixed on the left and right universal joint of the micromanipulator and alignment of the electrodes was conducted in the fusion dish (Figure 3.20 (i)). Subsequently, the pre-equilibrated couplets were transferred into the ZFM droplet and deposited between two vertical lines marked on the fusion dish. The donor cell-cytoplast couplet was sandwiched between the two electrodes (Figure 3.20 (ii)) and two direct current pulses (20-21 V, 15 µsec for caprine and 24 V, 15 µsec for bovine and gaur) were introduced to fuse the couplets. The couplets were washed in EMCARE^{TM} holding solution 5 times (15 minutes) and held in incubated holding medium for 1 hour (38.5°C, 5% CO_2) before assessing fusion. The couplets successfully fused were subjected to activation treatment.



Note: (photomicrograph (ii) original magnification: 100x)

Figure 3.21: Electrofusion. (i) Placement of electrodes and fusion dish on the micromanipulator; (ii) Position of donor karyoplast at 3 'clock, sandwiched between 2 electrodes.

3.3.7.6 Activation treatment

Two activation protocols were used in this study to generate cloned embryos and parthenotes namely:

- Protocol A: EtOH [7%] (5 min) followed with CD [1.25 µg/ml] + CHX [10 µg/ml] (5 h); for gaur iSCNT, bovine SCNT and bovine PA embryos production
- Protocol B: CaI [5 µM] (5 min) followed with 6-DMAP [2 mM] (4 h), developed by Shen *et al*. (2008); for caprine SCNT, iSCNT and PA embryos production

Protocol A was carried out by treating the fused couplets in EtOH (7%) droplets (7 droplets) for 5 minutes at room temperature. Subsequently, the fused couplets were washed in CD-CHX solution before culturing them in CD-CHX droplets (70 µl) for 5 hours at 38.5°C under a humidified atmosphere of 5% CO_2 in air.

Protocol B was carried out by culturing the fused couplets in CaI droplets for 5 minutes at 38.5°C under a humidified atmosphere of 5% CO_2 in air. The fused couplets were washed in IVC medium (mSOFaa or KSOMaa according to the experimental design) before culturing them in 6-DMAP droplets for 4 hours at 38.5°C under a humidified atmosphere of 5% CO_2 in air.

3.3.8 *In Vitro* Culture

In this study, three IVC protocols were used namely:

- Protocol I: mSOFaa medium for 8 days IVC (renewal of IVC medium was done at Day- 2, 4, 6, 7, 8 post-activation). This protocol was used in Experiments 3 and 4
- Protocol II: KSOMaa A medium for 8 days IVC (renewal of IVC medium was done at Day- 2, 4, 6, 7, 8 post-activation). This protocol (Treatment A) was used in Experiment 4
- Protocol III: KSOMaa A medium for the first 2 days of IVC followed by KSOMaa B medium for the subsequent 6 days of IVC (renewal of IVC medium was done at Day-2, 4, 6, 7, 8 post-activation). This protocol (Treatment B) was used in Experiments 4 and 5

The reconstructed oocytes were washed and cultured in equilibrated IVC medium according to the respective IVC protocols mentioned above at 38.5°C under a humidified atmosphere of 5% CO_2 in air. Assessment of the embryos' *in vitro* development (IVD) was conducted on the same day as the renewal of IVC medium was done.

3.3.9 Nucleic acid staining

In the present study, nucleic acid staining using Hoechst 33342 dye was conducted on caprine matured oocyte to observe its nuclear progression approaching meiotic maturation. This staining method was also used to stain cloned blastocyst to determine the cell number. Besides that, Hoechst 33342 dye was also used to stain the extruded polar body- cytoplasm material for the confirmation of positive enucleation.

Typically, the specimen to be stained were first washed in PBS(-) droplets (5 droplets; 100 µl/ droplet). Subsequently, the specimens were washed in 3 droplets of the fixative solution (100 µl/ droplet). The specimens were exposed to the fixative solution for 5 minutes. A glass slide was then wiped with EtOH (70%) and Vaseline-wax was placed onto the four corner of a box dimension (size of a cover slip) at the centre of the slide. The specimens were transferred onto the centre of the glass slide marked by the Vaseline-wax deposition and a cover slip was then mounted on it. Subsequently, Hoechst 33342 dye (~2 µl) was placed on the side of the mounted cover slip and by the capillary action, the dye was absorbed into the specimens. The 4 edges of the cover slip were finally sealed with adhesive (cutex). The specimen slide was labelled, wrapped with the aluminium foil and kept in the refrigerator (4°C) to be observed under the fluorescent microscope.

3.3.10 Embryo Transfer

Embryo transfer was carried out only on cloned caprine embryos using two approaches namely, oviductal transfer and uterine transfer. Cloned caprine embryos (4 to 8-cell) at Day-2 post activation were transferred into the oestrus synchronised pseudo-pregnant doe via oviduct transfer method, while cloned caprine embryos (compact morula to blastocyst) at Day-6 post activation were transferred via uterine transfer method.

The oestrus cycle of the recipient doe was synchronised according to the protocol shown in Figure 3.20. On the day of surgery, the ovaries of the recipient does were checked for the presence of corpus luteum (CL) via laparoscopic procedure similar to the LOPU procedure described in sub-section 3.3.6.1(f). Embryos were transferred to the oviduct or uterine ipsilateral to the corpus luteum.

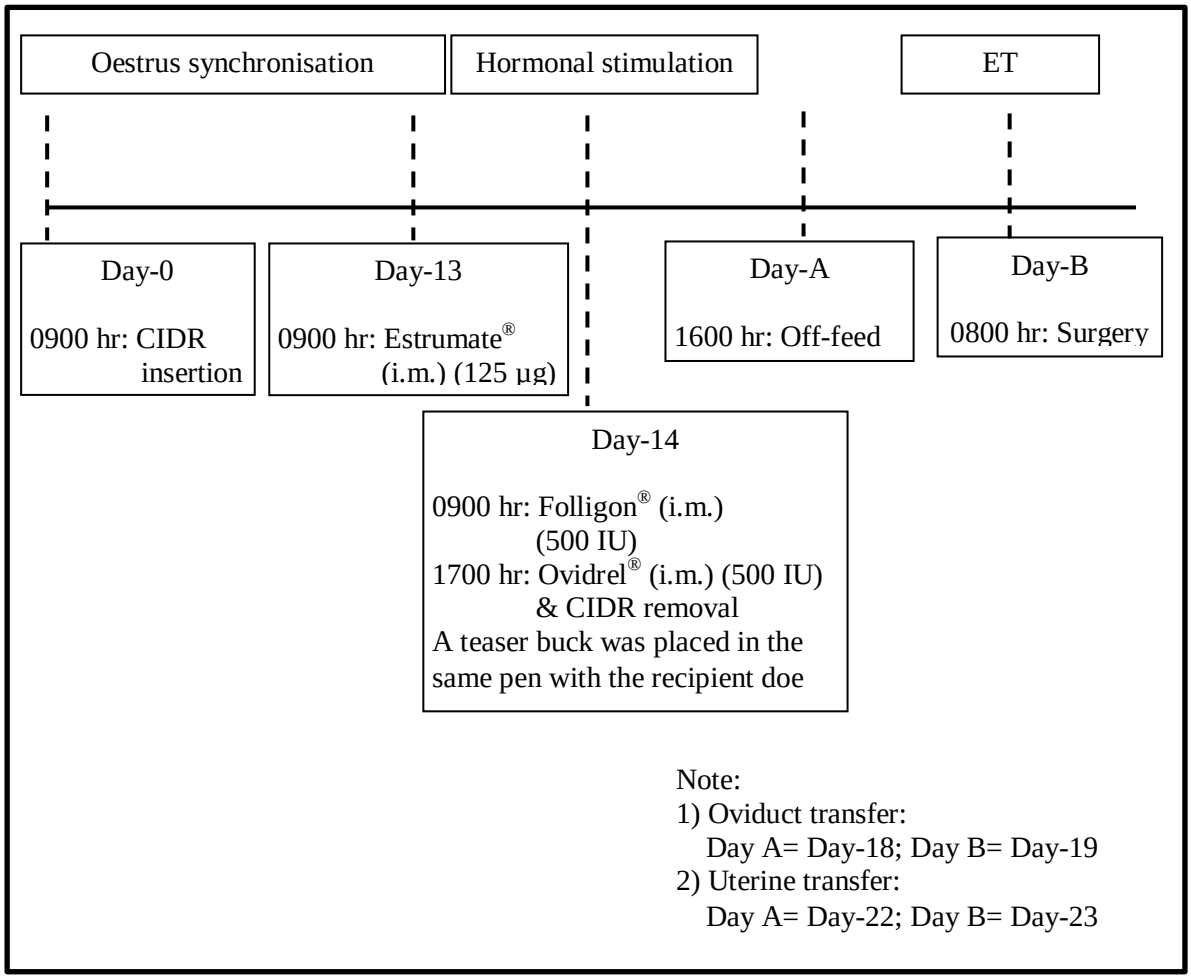


Figure 3.22: Protocol for recipient doe's oestrus synchronisation.

The oviduct transfer was carried out by making a mid-ventral incision (~5 to 6 mm) at the lower abdomen near the udder. The ovary with corpus luteum were located and exteriorised. Embryo transfer was carried out with a customised embryo transfer catheter (flexible polythene tubing, threaded through a 25-G hypodermic needle). Loading of the embryos into the embryo catheter was performed by the embryologist. Briefly, an insulin syringe (1 ml) that was filled with EMCARE^{TM} holding solution (0.3 ml) was connected to the embryo transfer catheter. The catheter was then flushed with the EMCARE^{TM} holding solution until 0.1 ml of the medium was left in the syringe. A small column of air was then aspirated into the catheter. Subsequently, 2 to 4 cloned embryos at 4-8 cell stages were aspirated into the catheter resulting in a 5 mm column containing the embryos and medium. A further 5 mm air column was made. The catheter was inserted through the ostium advancing the catheter as far as possible into the infundibulum of the oviduct and gently, the cloned embryos were released into the infundibulum by depressing the plunger of the syringe gradually. Once completed, the ovary was placed back into the cavity of the abdomen and the incision was sutured. The sutured incision area was sprayed with antiseptic and oxytetracycline (20 mg/kg body weight) was injected into the doe via i.m. once in every 4 days within the duration of 2 weeks to prevent post-surgical infection.

Uterine transfer is considered a less invasive surgical approach as the uterine horn was exteriorised through the small incision made for the access of the grasping forceps during laparoscopic detection of the CL. The uterine horn was clamped with a haemostat before piercing a hole on the uterine using a sterile paper clip. The cloned embryos were loaded in a Unopette attached to a syringe (1 ml). The method of loading the embryos is similar to the approach using the customised catheter mention in the previous paragraph. Once the loading completed, the Unopette tip was inserted through the hole introduced on the uterine and the embryos were released into the uterine slowly.

The uterine was released from the haemostat and the incision was sutured. The recipient does were given antibiotic injection described previously and their diet was well taken care.

Pregnancy diagnosis was conducted 35 days after embryo transfer. A real-time ultrasound scanner (Aloka SSD500V, Tokyo, Japan) equipped with 5.0 mHz linear array transducer for the transrectal approach was used. The transducer was coated with carboxymethylcellulose gel before used.

3.4 EXPERIMENTAL DESIGN

The present study of producing cloned goat embryos was the first attempt conducted in this laboratory, therefore the primary objective of this study was to develop a somatic cell nuclear transfer (SCNT) protocol to be served as a stepping stone for future study related to goat nuclear transfer research in this laboratory. There were two SCNT approaches investigated namely intraspecies SCNT (intraspSCNT) in which both the recipient oocytes and donor nucleus were derived from the same species and interspecies SCNT (interspSCNT) which describe that both recipient oocytes and donor nucleus derived from different species. This study focused not only on the aspect of SCNT, but also on the aspect of caprine superstimulation responses, sources of oocyte recovery, *in vitro* maturation (IVM) and *in vitro* culture (IVC) media for caprine embryos in some extent. The study was divided into five main experiments. The design of each experiment was described in the following section with a summary of experimental design flow chart and a methodology flow chart illustrated in Figure 3.21 and Figure 3.22, respectively.

3.4.1 Effect of Different Sources of Gonadotrophin on Caprine Superstimulation Responses (Experiment 1)

The objectives of this experiment was to evaluate the effect of: a) two different sources of gonadotrophin namely Folligon® and Folltropin®-V on caprine ovarian responses and b) three repeated stimulation and OR cycles on the ovarian responses. The parameters measured for both objectives were the number of follicles recruited, number of oocyte retrieved, oocyte retrieval rate and the percentage of oocytes retrieved after grading as a

mean of oocyte quality evaluation. Classification of the oocyte Grade was according to the cumulus cell investment and oocyte morphology described in Table 3.38.

A total of 33 does (aged 18 to 24 months) were synchronised (CIDR device and Estrumate®) and stimulated in this experiment. Out of the 33 synchronised does, 18 does were stimulated with Folligon® and Ovidrel®; while the remaining 15 does were stimulated with Folltropin®-V and Ovidrel® according to the regime designated in Figure 3.8. The OR surgery was conducted at the onset of oestrus by LOPU technique. The number of follicles recruited, quantity and quality of oocytes retrieved from ovaries stimulated with Folligon® versus Folltropin®-V and from OR1 to OR3 were analysed by analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT).

3.4.2 Effect of Different Sources of Caprine Oocytes on the Oocyte Yield, Grades and Maturation Performance (Experiment 2)

The experiment was designed to compare the quantity, quality and maturation rate of oocytes recovered from live does by LOPU versus slicing of abattoir-derived ovaries. In this experiment, oocytes of Grade E was not taken into consideration as generally abattoir source produces abundant Grade E oocytes and picking-up of this oocytes is rather time-consuming. Generally the live does were hormonally stimulated with pFSH regimen and the interval between the hormonal treatment and LOPU used was 70 to 72 hours.

Prior evaluating the maturation competency of LOPU- versus abattoir-derived oocytes, determination of an optimum IVM duration range for both oocyte sources was

conducted. Oocytes from LOPU and abattoir sources were subjected to IVM and the maturation rates were recorded at the interval of 3 hours starting from 15 hours to 27 hours post-IVM. The IVM durations selected (15 hour to 27 hours) were based on the range of IVM duration (16 to 27 hours) that was frequently used by other researchers (Cognié *et al*., 2003; Wang *et al*., 2003; Jimenez-Macedo *et al*., 2006; Rahman *et al*., 2008) for caprine oocyte maturation regardless of their source of oocytes, superovulation protocols and the formulation of IVM medium. Observation on the morphology of PB-1 and the localisation of MII spindle and PB-1 for all the matured oocytes at 15, 18, 21, 24 and 27 hours were recorded. The optimum IVM duration range was determined based on the duration in which the maturation rate recorded is reasonably high, majority of the morphology of PB-1 is not fully extruded, less fragmentation and the location of the MII spindle is closely adjacent to the PB-1. The optimum IVM duration range determined in this sub-experiment was employed in the subsequent experiments that involve the IVM of caprine oocytes.

A total of 14 does (28 ovaries) were used for LOPU. On the other hand, 56 ovaries from 28 does were collected from a local abattoir. Experimental does for LOPU were subjected to oestrus synchronisation and superstimulation using pFSH regime (Figure 3.8: Protocol 2). The recovered COCs from both LOPU- and abattoir-derived ovaries were graded and subjected to the same IVM medium (Table 3.8) incubated in the presence of CO_2 (5%) at 38.5°C. The COCs were denuded at the optimum IVM duration using hyaluronidase to select the MII oocytes. A small portion of the MII oocytes were stained with Hoechst 33342 dye to observe the location of the MII spindle from PB-1.

The quantity of oocytes retrieved and the maturation rates obtained from LOPU versus abattoir sources were analysed by the analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT).

3.4.3 Production of Cloned Bovine and Gaur Embryos via Intraspecies and Interspecies SCNT Approaches: A Preliminary Study for Caprine SCNT Research (Experiment 3)

This experiment was conducted during a 4 months (August 2009 to December 2009) training programme in Embryo and Stem Cell Research Center (ESRC), Suranaree University of Technology, Thailand. Since the protocol for bovine intraspecies SCNT (intraspSCNT) and gaur interspecies SCNT (interspSCNT) were rather established compared to other animal model in ESRC and worldwide, as well as its physiology is closely related to caprine, thus this experiment was carried out as a scaffold for the development of caprine SCNT protocol in this laboratory. While adapting the SCNT protocol from this experiment, a comparison was made in the efficiency between intraspSCNT versus interspecies interspSCNT approach. The efficiency between both approaches was measured in terms of the fusion rate, cleavage rate and the cloned embryo *in vitro* developmental (IVD) rate from 2-cell stage to hatched blastocyst stage. The fusion rate was calculated based on the number of fused cells divided by the number of embryos that passed through the electric current generated by the fusion machine and the cleavage rate was calculated from the number of cleaved embryo divided by the number of fused embryos. The IVD rate was calculated based on the number of embryos at each developmental stages divided by the number of fused embryos subjected to *in vitro* culture (IVC).

A total of 181 and 203 matured bovine oocytes were used in the bovine intraspSCNT and gaur interspSCNT experiment. Bovine oocytes were collected from the local abattoir and transported to the laboratory in 0.9% NaCl at 35 to 37°C. The COCs were collected by aspiration of follicles 2-8 mm in diameter using a 21 gauge needle attached to a 10 ml syringe. COCs were cultured in IVM medium for 22 to 24 hours. After IVM, the cumulus cells were mechanically removed from the oocytes by repeated pipetting using fine-tip pipette in mDPBS supplemented with 0.2% hyaluronidase. Matured oocytes with the presence of PB1 were subjected to the SCNT manipulation that consists of 3 main steps: a) enucleation via squeezing technique, b) injection of donor karyoplast into the perivitelline space of the enucleated oocytes and c) electrofusion. Matured bovine oocytes were subjected to cytochalasin B (CB) treatment for 5 minutes prior enucleation. Complete enucleation was then confirmed by staining the squeezed out cytoplasm with Hoechst 33342 (5 µg/ml) and visualizing the stained nucleus in the cytoplasm under the fluorescence microscope. Bovine and gaur ear fibroblast (EF) cells at passage 3 (P3) to P5 were used as the donor karyoplast in the production of bovine intraspSCNT and gaur interspSCNT embryos, respectively. Both bovine or gaur karyoplast- bovine cytoplast couplets were fused in ZFM medium (Table 3.18) using two direct current pulses (24 V, 15 µsec) and both successfully fused reconstructed oocytes were activated using the same chemical activation treatment of EtOH (7%), 5 minutes followed by CD-CHX, 5 hours cultured under a humidified atmosphere of 5% CO_2 in air at 38.5°C. The bovine intraspSCNT and gaur interspSCNT reconstructed oocytes were cultured in mSOFaa medium at 38.5°C under humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 for 2 days. Embryos at the 8-cell stage were selected and co-cultured with bovine oviductal epithelial cells in mSOFaa medium at 38.5°C under a humidified atmosphere of 5% CO_2 in air for an additional 5 days (7 days

in total). Half of the IVC medium volume was replaced daily from Day 4 onwards and development of the embryos was recorded.

The fusion rate, cleavage rate and IVD rates obtained from bovine intraspSCNT approach versus gaur interspSCNT approach were analysed by the analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT).

3.4.4 Improvement on the *In Vitro* Cloned Caprine Embryos Production by Considering the Effects of Maturation Duration, Activation Treatment and *In Vitro* Culture Protocol (Experiment 4)

As an effort to develop a caprine intraspSCNT protocol for the present laboratory setting and to contribute new findings to the field of caprine intraspSCNT, three sub-experiments were conducted to evaluate the effects of: a) maturation duration, b) activation treatment and c) IVC medium on the developmental competency of cloned caprine intraspSCNT embryos.

In the first sub-experiment, the effect of two different maturation duration range, 18 to 22 hours and 23 to 27 hours on the IVD competency of cloned caprine embryos using LOPU derived oocytes was evaluated. A total of 83 and 109 caprine oocytes derived from stimulated ovaries were subjected to IVM for a duration range of 18 to 22 hours and 23 to 27 hours, respectively. Comparison was made in terms of the percentage of maturation, successful enucleation rate, cleavage rate and IVD rate from 2-cell to morula stage between oocytes derived from the two IVM duration range tested. The matured caprine oocytes were enucleated and reconstructed with caprine ear fibroblast (EF) cell (P3 to P5) using the similar protocol described for bovine

intraspSCNT in Experiment 3. The successfully fused couplets were chemically activated using calcium ionophore [5 µM] (5 min) followed with 6-DMAP [2 mM] (4 h) adapted from Shen *et al*. (2008). In this experiment, the reconstructed caprine oocytes were cultured in mSOFaa medium without co-culture with bovine oviductal epithelial cells at 38.5°C under a humidified atmosphere of 5% CO_2 in air. Changing of medium was done at Day-2, 4, 5, 6 and 7 post- activation. The IVD of the cloned embryos was recorded when changing of IVC medium was conducted. The optimum IVM duration range determined in this sub-experiment was employed in the subsequent caprine intraspSCNT experiment that utilised LOPU-derived oocytes.

In the second sub-experiment, the effect of two different sequential chemical activation treatments, namely:

i. Protocol A: EtOH [7%] (5 minutes) followed with CD [1.25 µg/ml] + CHX [10 µg/ml] (5 hours), adapted from Experiment 3
ii. Protocol B: CaI [5 µM] (5 minutes) followed with 6-DMAP [2 mM] (4 hours), developed by Shen *et al*. (2008) on the IVD competency of caprine intraspSCNT embryos was evaluated.

A total of 67 and 59 fused reconstructed caprine oocytes were subjected to chemical activation using protocol A and B, respectively. The activated reconstructed caprine oocytes were cultured in mSOFaa medium at 38.5°C under a humidified atmosphere of 5% CO_2 in air for 8 days and changing of medium was done similarly as the first sub-experiment. The IVD of the cloned embryos was recorded from 2-cell stage onwards up to Day 8.

The third sub-experiment was conducted to compare the efficacy between mSOFaa medium and KSOMaa medium (denoted as KSOMaa A in this experiment) in supporting the IVD of cloned caprine embryos. Besides that the effect of using the two-

step culture system (Treatment B) by increasing the glucose concentration in KSOMaa medium (denoted as KSOMaa B in this experiment) at Day 2 culture compared to the one-step culture system using the classical KSOMaa medium (Treatment A) on the IVD competency of cloned caprine embryos was also evaluated in the fourth sub-experiment.

A total of 77 and 70 reconstructed caprine oocytes activated using CaI [5 µM] (5 minutes) followed with 6-DMAP [2 mM] (4 hours) were subjected to IVC using mSOFaa and KSOMaa A, respectively. In a separate experiment, a total of 122 and 131 reconstructed caprine oocytes were cultured in IVC Treatment A and Treatment B, respectively. In IVC Treatment A, reconstructed caprine oocytes were cultured in classical KSOMaa A medium for 8 days, while for IVC Treatment B, reconstructed caprine oocytes were cultured in KSOMaa A medium for the first 2 days and subsequently in KSOMaa supplemented with glucose to the final concentration of 2.78 mM (KSOMaa B) for another 6 days. The IVD of the cloned embryos was recorded from 2-cell stage onwards up to Day 8 during the renewal of IVC medium at Day 2, 4,5,6,7 and 8.

3.4.5 Efficacy of Producing Cloned Caprine Embryos Using Intraspecies versus Interspecies SCNT Approaches (Experiment 5)

This experiment was conducted to evaluate the efficacy of producing cloned caprine embryos using intraspSCNT and interspSCNT approaches and comparison was made between both approaches in terms of the maturation rate, enucleation rate, fusion rate, cleavage rate and IVD rate. The efficacy of each approach was evaluated by comparing

the IVD rate of reconstructed cloned caprine embryos of intraspSCNT and interspSCNT versus caprine and bovine parthenotes (as control), respectively. Besides that, the quality of caprine intraspSCNT, interspSCNT and PA hatched blastocyst were investigated by enumerating the number of cells in the hatched blastocyst obtained at Day- 8. An attempt to evaluate the implantation competency of caprine intraspSCNT and interspSCNT embryos was conducted by carrying out embryo transfer.

A total of 235 caprine oocytes retrieved from LOPU- and abattoir-derived ovaries were used in the production of caprine intraspSCNT embryos and parthenotes. On the other hand, 434 bovine oocytes retrieved from abattoir-derived ovaries were used in the caprine interspSCNT experiment and in the production of bovine parthenotes. In order to evaluate the efficacy of both the NT approaches, all the oocytes retrieved either from caprine or bovine were subjected to the same formulated IVM medium and the reconstructed caprine intraspSCNT, interspSCNT and parthenogenesis activated (PA) oocytes were subjected to the same chemical activation protocol B (CaI [5 µM], 5 min followed with 6-DMAP [2 mM], 4 hr) as well as cultured in the same IVC Treatment B at 38.5°C under a humidified atmosphere of 5% CO_2 in air for 8 days.

A total of 4 to 5 hatched blastocysts derived from each IVP approaches, namely caprine intraspSCNT, interspSCNT, PA and bovine PA were stained with Hoechst 33342. The quality of the hatched blastocysts obtained was evaluated by enumerating the number of stained nuclei of the cells that presence.

Embryo transfer attempts were conducted to evaluate the post-implantation competency of the caprine intraspSCNT and interspSCNT embryos. A total of 12 female does were subjected to oestrus synchronisation and stimulation according to the regime depicted in Figure 3.20. Embryo transfer was conducted on the recipient does that were successfully synchronised and stimulated with the presence of at least 1

corpus luteum (CL). Two embryo transfer approaches namely oviductal transfer and uterine transfer were used for the 2-cell to 8-cell and 8-cell to morula stage cloned caprine embryos (intraspSCNT or interspSCNT), respectively. Pregnancy of the recipients was diagnosed at 35 days by a real time ultrasound scanner (SSD500V; Aloka, Japan) equipped with transrectal probe

3.5 STATISTICAL ANALYSES

Majority of the data collected in all the experimental designs in this present study were presented in mean±standard error of means (mean±SEM) and the data were analysed using one-way analysis of variance (ANOVA). The effects of different factors on donor does hormonal stimulation, OR, IVM and embryo development following nuclear transfer, specified in each experiment were compared and the significant differences between the means were further analysed using Duncan Multiple Range Test (DMRT) to show specific differences among the factors on the parameter measured and $P<0.05$ was considered significant. The analysis was carried out with SPSS (Statistical Packages for Social Sciences) for windows, version 17.0, SPSS Inc (2010), USA.

Preparation of Donor Does (Oestrus Synchronisation & Superstimulation) for Oocyte Retrieval via LOPU (Experiment 1)

Factors:

a) Source of gonadotrophin (PMSG vs. pFSH)
b) Number of OR cycles (OR1 vs. OR2 vs. OR3)

Parameters:

- No. of follicles per ovary
- No. of oocytes retrieved per ovary
- Oocyte retrieval rate
- Percentage of oocytes retrieved according to oocyte quality (Grade A, B, C, D, E)

↓

Oocyte Retrieval & *In Vitro* Maturation (IVM) of Oocytes Derived from Different Sources (Experiment 2)

Factors:

a) Source of oocytes (LOPU vs. Abattoir)
b) IVM durations (15, 18, 21, 24 and 27 hours)

Parameters:

- No. of oocytes retrieved per doe
- Percentage of oocytes retrieved according to oocyte quality (Grade A, B, C and D)
- Percentage of matured oocytes

Observations:

- Morphology of first polar body (PB-1)
- Localisation of MII spindle and PB-1

↓

Production of Bovine intraspSCNT & Gaur interspSCNT Embryos: Preliminary Study for Caprine SCNT (Experiment 3)

Factors:

a) Approaches of SCNT (Bovine intraspSCNT vs. Gaur interspSCNT)

Parameters:

- Percentage of successful enucleation
- Fusion rate
- Cleavage rate
- IVD rate (2-, 4-, 8- cell, morula, blastocyst and hatched blastocyst)

↓

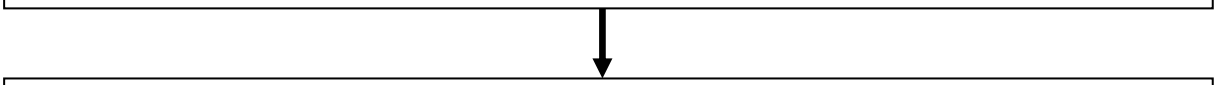
Production of Caprine intraspSCNT embryos: Effort to Develop Caprine intraspSCNT Protocol (Experiment 4)

Factors:

a) Optimum IVM durations for LOPU-derived oocytes (18-22 hr vs. 23-27 hr)
b) Chemical activation protocols (Protocol A: 7% EtOH + CD-CHX vs. Protocol B: CaI + 6-DMAP)
c) IVC medium (mSOFaa vs. KSOMaa)
d) Modification of glucose concentration in KSOMaa medium (Treatment A IVC: IVC in KSOMaa for 8 days vs. Treatment B IVC: IVC in KSOMaa for 2 days + KSOMaa with final concentration of 2.78 mM glucose for another 6 days)

Parameters:

- Maturation rate
- Percentage of successful enucleation
- Fusion rate
- Cleavage rate
- IVD rate (2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst)



Production of Caprine intraspSCNT and interspSCNT embryos (Experiment 5)

Factors:

a) Type of IVP embryos (Cloned embryos vs. parthenotes)
b) Nuclear transfer approaches (intraspSCNT vs. interspSCNT)

Parameters:

- Maturation rate
- Percentage of successful enucleation
- Fusion rate
- Cleavage rate
- IVD rate (2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst)
- Number of cells in hatched blastocyst
- Pregnancy rate to evaluate implantation competency of cloned caprine embryos after embryo transfer

Figure 3.23: Flow chart of experimental design.

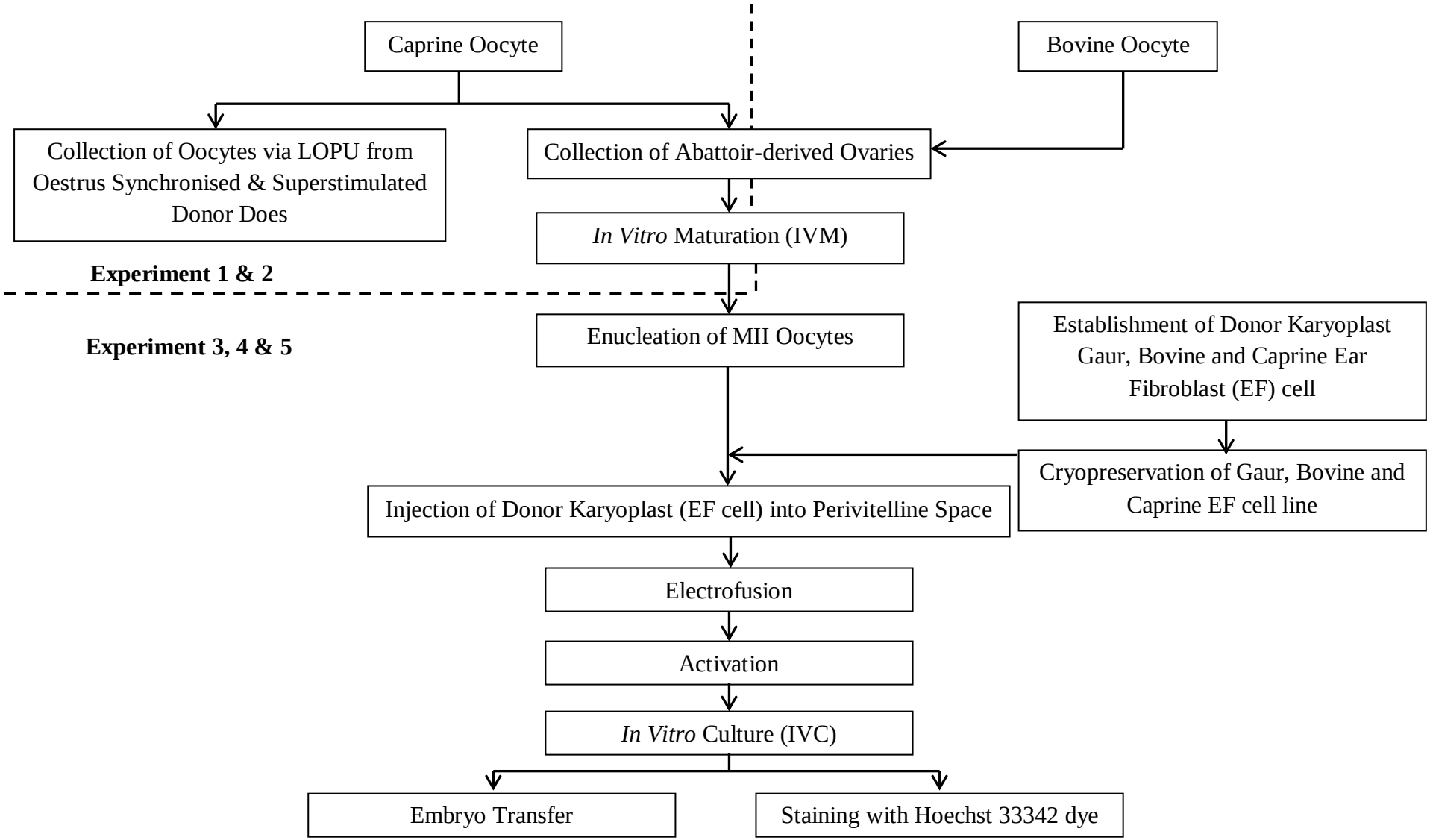


Figure 3.24: Flow chart of methodology.

Chapter 4

4.0 RESULTS

Chapter 4

4.0 RESULTS

4.1 EFFECT OF DIFFERENT SOURCES OF GONADOTROPHIN ON CAPRINE SUPERSTIMULATION RESPONSES (EXPERIMENT 1)

In order to study the effect of PMSG (Folligon®) and pFSH (Folltropin®-V) as superstimulation hormones on caprine LOPU-derived oocyte retrieval rate and quality, a total of 18 healthy does were randomly selected for oestrus synchronisation and superstimulation using single dose injection of PMSG (1200 IU/doe) for oocyte retrieval (OR) cycle 1 and 1500 IU/doe for OR2 and OR3, while another 15 healthy does were subjected to multiple dose injection of pFSH (overall dosage: 70 mg/doe) in each OR cycle for all the 3 OR cycles. Both stimulation protocols using PMSG (Folligon®) and pFSH (Folltropin®-V) were coupled with the administration of hCG (Ovidrel®) and does were recuperated for an interval of 3 months between the OR cycles. The duration between PMSG or pFSH injection and the onset of LOPU was standardised at the duration range of 64 to 67 hours and the cumulus oocyte complexes (COCs) were retrieved via aspiration from antral follicles of 2 to 3 mm or bigger in diameter during laparoscopic oocyte pick-up (LOPU) procedure. The oocytes yielded were graded according to cumulus cell investment and oocyte morphology, presented in Figure 4.1)

The effect of PMSG and the number of OR cycles on the caprine ovarian response were summarised in Table 4.1. Does stimulated with PMSG resulted in an average of 16.4 follicle formation and 11 oocytes yielded per doe in the three OR cycles measured. The effect of PMSG on the ovarian response seemed to decrease when

approaching OR3 as the number of follicles and oocytes retrieved (an average of 9.7 and 6.3, respectively) decreased significantly ($P<0.05$) compared to its previous cycle (16 and 12.1, respectively). Even though the oocyte yield decreased in OR3, the quality of the oocytes was not affected as the percentages of oocytes for Grades A, B and C retrieved did not differ significantly ($P>0.05$) from the previous two OR cycles. In OR1 and OR2, majority of the oocytes retrieved were of Grades A, B and C with the percentages ranged from 22.3 to 32.3%, and no significant differences ($P>0.05$) were observed among the percentages of these oocyte grades yielded within their respective OR cycles. As the number of OR cycles increased (OR1 to OR3), the percentages of Grades D and E oocyte yield increased correspondingly. Significant ($P<0.05$) increment in the percentages of Grades D and E oocytes was observed particularly between OR1 (8.9% and 1.4%, respectively) vs. OR3 (23.6% and 13.8%, respectively).

The effect of pFSH and the number of OR cycles on the caprine ovarian response were depicted in Table 4.2. The stimulation effect of pFSH apparently similar throughout the three OR cycles. Even though the number of follicle formation and number of oocytes oocytes retrieved in the first OR cycle was the highest and declined steadily for the consecutive OR cycles; however, this was not statistically significant ($P>0.05$). An average of 15.9 follicles was observed and 12.1 oocytes retrieved per doe in the three OR cycles. In terms of the oocyte quality, the average percentages of Grades A, B, C, D and E oocytes retrieved in three OR cycles were 35.8, 35.2, 18.4, 8.8 and 1.9%, respectively. No significant differences ($P>0.05$) in the percentages of oocyte Grades A, B, C, D and E were observed between the three OR cycles. In OR1 and OR3, the pFSH gave a positive effect on oocyte quality that resulted in significantly ($P<0.05$) higher percentages of Grades A (36.2 and 41.3%, respectively) and B (41.4 and 26.8%, respectively) oocytes retrieved compared to Grades C, D and E.

Table 4.1: Number and percentages (mean±SEM) of oocytes retrieved from caprine superstimulated with PMSG according to OR cycle and oocyte grade

OR cycle	No. of ovaries	No. of follicles per ovary	No. of oocytes retrieved per ovary	*Oocyte retrieval rate	Percentage of oocytes retrieved according to oocyte quality (n)				
					Grade A	Grade B	Grade C	Grade D	Grade E
OR1	36	8.83 ± 0.5^{b} (308/36)	5.61 ± 0.6^{ab} (202/36)	60.71 ± 4.3^{a} (202/308)	$29.30\pm4.6^{a,y}$ (55/202)	$28.17\pm5.1^{a,y}$ (44/202)	$32.25\pm4.5^{a,y}$ (78/202)	$8.88\pm2.2^{a,x}$ (22/202)	$1.39\pm1.4^{a,x}$ (3/202)
OR2	18	8.00 ± 0.6^{b} (144/18)	6.06 ± 0.5^{b} (109/18)	76.70 ± 4.3^{a} (109/144)	$28.49\pm4.3^{a,z}$ (34/109)	$29.49\pm4.1^{a,z}$ (31/109)	$22.25\pm4.8^{a,yz}$ (25/109)	$11.54\pm3.5^{ab,xy}$ (12/109)	$8.24\pm4.0^{ab,x}$ (7/109)
OR3	6	4.83 ± 0.8^{a} (29/6)	3.17 ± 0.3^{a} (19/6)	72.36 ± 10.5^{a} (19/29)	$19.45\pm10.7^{a,x}$ (4/19)	$16.67\pm7.5^{a,x}$ (3/19)	$26.39\pm9.2^{a,x}$ (5/19)	$23.61\pm11.9^{b,x}$ (4/19)	$13.89\pm9.0^{b,x}$ (3/19)
Average	20 (60/3)	8.18±0.4 (481/60)	5.5±0.4 (330/60)	66.7±3.2 (330/481)	28.1±3.2 (93/330)	27.4±3.3 (78/330)	28.7±3.2 (108/330)	11.2±2.1 (38/330)	4.7±1.8 (13/330)

[ab]Means with different superscripts in a column within a group were significantly different (P<0.05).
[xyz]Means with different superscripts in a row within a group were significantly different (P<0.05).
*Oocytes retrieval rate refer to the number of oocytes retrieved upon number of follicles observed.

Table 4.2: Number and percentage (mean±SEM) of oocytes retrieved from caprine superstimulated with pFSH according to OR cycle and oocyte grade

OR cycle	No. of ovaries	No. of follicles per ovary	No. of oocytes retrieved per ovary	*Oocyte retrieval rate	Percentage of oocytes retrieved according to oocyte quality (n)				
					Grade A	Grade B	Grade C	Grade D	Grade E
OR1	30	8.20±0.5^{a} (248/30)	6.23±0.6^{a} (187/30)	74.28±4.1^{a} (187/248)	36.23±4.0^{a,z} (63/187)	41.38±3.8^{a,z} (78/187)	16.44±3.6^{a,y} (29/187)	5.18±1.6^{a,x} (14/187)	0.80±0.5^{a,x} (3/187)
OR2	10	7.60±0.7^{a} (76/10)	5.70±0.5^{a} (57/10)	76.45±5.2^{a} (57/76)	32.50±9.0^{a,y} (17/57)	20.02±5.4^{a,xy} (13/57)	25.12±6.1^{a,y} (14/57)	16.52±5.0^{a,xy} (10/57)	5.83±3.9^{a,x} (3/57)
OR3	4	7.00±0.9^{a} (28/4)	5.75±0.8^{a} (23/4)	82.99±7.1^{a} (23/28)	41.25±6.6^{a,y} (10/23)	26.79±11.8^{a,y} (6/23)	15.72±6.0^{a,xy} (4/23)	16.25±9.9^{a,xy} (3/23)	0.00±0.0^{a,x} (0/23)
Average	14.7 (44/3)	7.95±0.4 (352/44)	6.07±0.4 (267/44)	75.56±3.1 (267/352)	35.84±3.4 (90/267)	35.2±3.3 (97/267)	18.35±2.9 (47/267)	8.8±1.9 (27/267)	1.9±0.9 (6/267)

^{ab}Means with different superscripts in a column within a group were significantly different (P<0.05).
^{xyz}Means with different superscripts in a row within a group were significantly different (P<0.05).
*Oocytes retrieval rate refer to the number of oocytes retrieved upon number of follicles observed.

The results on the effect of PMSG vs. pFSH treatment on caprine ovarian response in each OR cycle are shown in Table 4.3. No significant difference ($P>0.05$) was observed in both the number of follicles and number of oocytes retrieved from does stimulated with either PMSG or FSH treatment in OR1 and OR2. However in OR3, the average number of oocytes retrieved from a doe stimulated with PMSG (6.4) was significantly ($P<0.05$) lower from a doe stimulated with pFSH (11.6).

In terms of oocyte quality, significant difference ($P<0.05$) between oocyte quality derived from ovaries stimulated with PMSG vs. pFSH was only observed in OR1. Caprine ovaries stimulated with pFSH produced significantly higher percentage of Grade B oocyte (41.4%) compared to PMSG stimulated ovaries (28.2%) in OR1. On the other hand, percentage of Grade C oocytes (32.3%) yielded was significantly higher from PMSG stimulated ovaries compared to pFSH stimulated ovaries (16.4%). Both PMSG and pFSH stimulations did not result in a high percentages of Grades D and E oocyte yielded in OR1, in which this was a rather favourable effect.

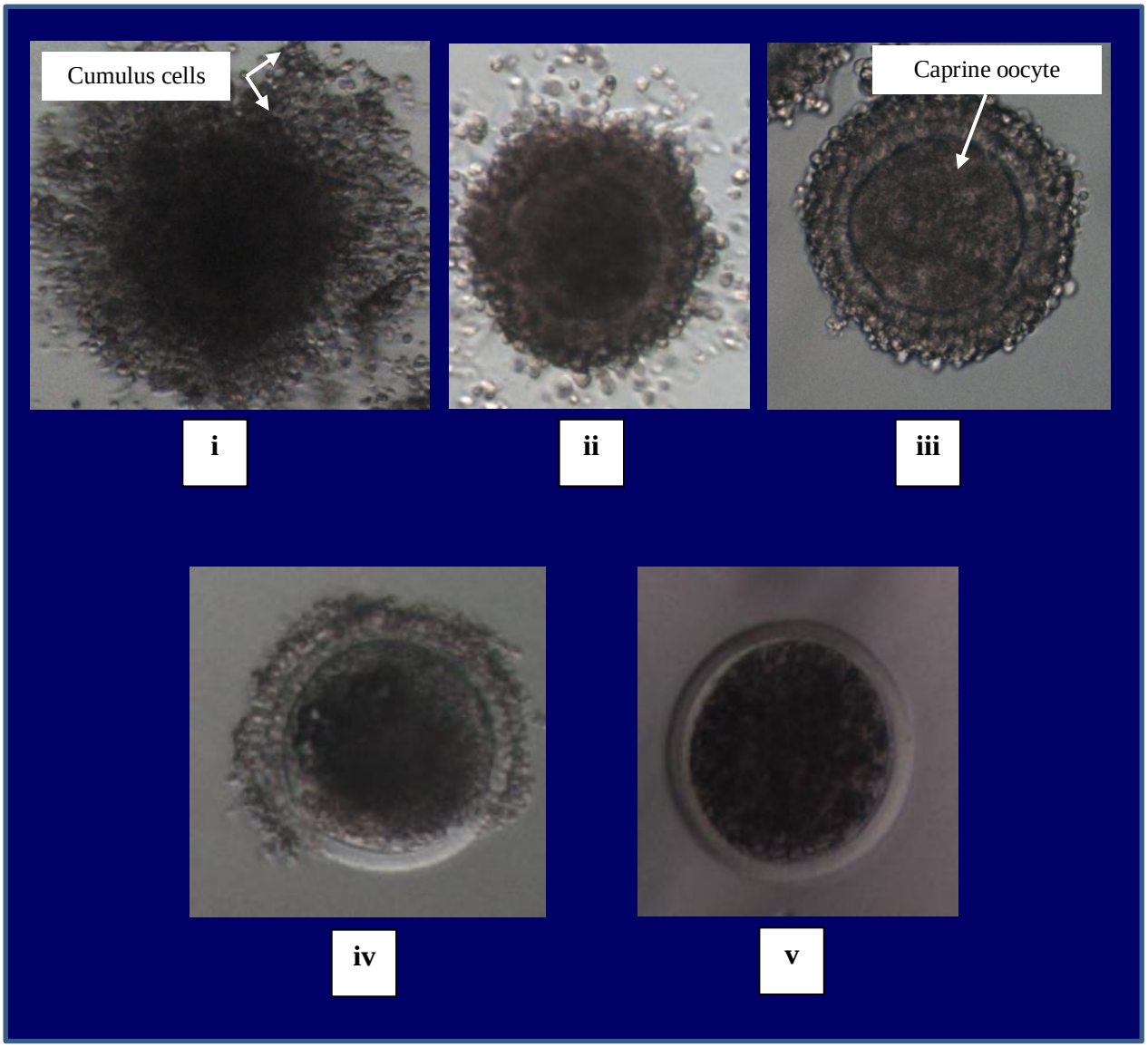
In OR2, no significant difference was observed in the percentages of oocyte yielded in each oocyte Grade between ovaries stimulated with PMSG vs. pFSH. Majority of the oocytes yielded from PMSG and pFSH stimulated ovaries distributed in the categories of Grades A, B and C.

In OR3, no significant difference was observed in the quality of oocytes yielded from PMSG vs. pFSH stimulated ovaries. Majority of the oocytes yielded from PMSG stimulated ovaries distributed in the categories of Grades A, C and D, while for pFSH stimulated ovaries majority of the oocytes were of Grades A and B.

Table 4.3: Number and percentage (mean±SEM) of oocytes retrieved from caprine superstimulated with PMSG versus pFSH according to OR cycle and oocyte grade

OR cycle	Type of hormone	No. of follicles per ovary	No. of oocytes retrieved per ovary	Percentage of oocytes retrieved according to oocyte quality (n)				
				Grade A	Grade B	Grade C	Grade D	Grade E
OR1	PMSG	8.8±0.5[a] (308/36)	5.6±0.6[a] (202/36)	29.3±4.6[a] (55/202)	28.2±5.1[a] (44/202)	32.3±4.5[b] (78/202)	8.9±2.2[a] (22/202)	1.4±1.4[a] (3/202)
	pFSH	8.2±0.5[a] (248/30)	6.2±0.6[a] (187/30)	36.2±4.0[a] (63/187)	41.4±3.8[b] (78/187)	16.4±3.6[a] (29/187)	5.2±1.6[a] (14/187)	0.8±0.5[a] (3/187)
OR2	PMSG	8.0±0.6[a] (144/18)	6.1±0.5[a] (109/18)	28.5±4.3[a] (34/109)	29.5±4.1[a] (31/109)	22.2±4.8[a] (25/109)	11.5±3.5[a] (12/109)	8.2±4.0[a] (7/109)
	pFSH	7.6±0.7[a] (76/10)	5.7±0.5[a] (57/10)	32.5±9.0[a] (17/57)	20.0±5.4[a] (13/57)	25.1±6.1[a] (14/57)	16.5±4.9[a] (10/57)	5.8±3.9[a] (3/57)
OR3	PMSG	4.8±0.8[a] (29/6)	3.2±0.3[a] (19/6)	19.4±10.7[a] (4/19)	16.7±7.5[a] (3/19)	26.4±9.2[a] (5/19)	23.6±11.9[a] (4/19)	13.9±9.0[a] (3/19)
	pFSH	7.0±0.9[a] (28/4)	5.8±0.8[b] (23/4)	41.3±6.6[a] (10/23)	26.8±11.8[a] (6/23)	15.7±6.0[a] (4/23)	16.3±9.9[a] (3/23)	0.0±0.0[a] (0/23)

[ab]Means with different superscripts in a column within a group were significantly different (P<0.05).



Note: (photomicrographs original magnification: 200x)

Figure 4.1: Caprine oocyte graded according to cumulus cells (CCs) investment and morphology of oocyte. (i) Grade A; (ii) Grade B; (iii) Grade C; (iv) Grade D; (v) Grade E.

4.2 EFFECT OF DIFFERENT SOURCES OF CAPRINE OOCYTES ON THE OOCYTE YIELD, GRADES AND MATURATION PERFORMANCE (EXPERIMENT 2)

This present study was conducted to investigate the effect of two different sources of caprine oocytes namely the LOPU and abattoir sources on the quantity and quality of oocytes yielded. Besides that, the optimum IVM time range for oocytes derived from both LOPU and abattoir sources were determined and the meiotic competence of the oocytes was evaluated.

4.2.1 Effect of Two Different Sources of Caprine Oocytes on the Quantity and Quality

Caprine oocytes used in this experiment were obtained from two different sources, namely retrieval from live does via LOPU and ovaries of slaughtered does from local abattoirs. Oocyte retrieval via LOPU procedure was carried out by aspirating only follicles of 2 to 3 mm or bigger in diameter; however, this could not be maintained in the case of abattoir-derived ovaries as during slicing, smaller follicles (<2 to 3 mm) could not be ignored. Evaluation on the oocyte quality from both sources did not include the percentage of Grade E oocytes.

The results for the oocyte yield and its quality from LOPU procedure and abattoir-derived ovaries are summarised in Table 4.4. A total of 154 oocytes were retrieved from 28 ovaries via LOPU and 549 oocytes from 56 sliced abattoir-derived ovaries. The oocyte recovery per doe was significantly ($P<0.05$) higher in abattoir (20.2) than LOPU (11) source. Abattoir source yielded higher oocyte recovery; however, better quality of oocytes was retrieved from LOPU source. This was

vividly apparent in the recovery of a significantly ($P<0.05$) higher proportion of Grade A oocytes retrieved from LOPU (39.2%) compared to abattoir source (18.4%). In addition, a significantly ($P<0.05$) lower proportion of Grade C (15.6% from LOPU vs. 33.8% from abattoir) and Grade D oocytes (5.0% from LOPU vs. 15.5% from abattoir) were retrieved from LOPU compared to the abattoir source. When considering different grades in each source, significantly ($P<0.05$) higher proportion of Grades A and B oocytes were retrieved from LOPU followed by Grades C and D. On the other hand, significantly ($P<0.05$) higher proportions of Grades B and C oocytes were retrieved from abattoir source followed by Grades A and D.

Table 4.4: Percentage (%, mean±SEM) of caprine oocytes retrieved from LOPU- versus abattoir-derived ovaries

Oocyte source	No. of replicates	No. of does (ovaries) used	No. of oocytes retrieved per doe	Percentage of oocytes retrieved according to oocyte quality (n)			
				Grade A	Grade B	Grade C	Grade D
LOPU	14	14 (28)	11.0±0.86[a] (154/14)	39.2±4.1[b,z] (58/154)	40.1±2.8[a,z] (63/154)	15.6±3.8[a,y] (24/154)	5.0±1.8[a,x] (9/154)
Abattoir	6	28 (56)	20.2±1.2[b] (549/28)	18.4±2.0[a,x] (104/549)	32.3±2.1[a,y] (175/549)	33.8±2.5[b,y] (182/549)	15.5±2.8[b,x] (88/549)

[ab]Means with different superscripts in a column within a group were significantly different (P<0.05).
[xyz]Means with different superscripts in a row within a group were significantly different (P<0.05).

4.2.2 Determination of an Optimised IVM Time Range for Caprine Oocytes Derived from Two Different Sources

The results for the determination of an optimal *in vitro* maturation (IVM) duration range for LOPU and abattoir derived caprine oocytes are depicted in Table 4.5. Maturation rate was measured by observing the presence of the first polar body (PB-1) at five IVM durations (15, 18, 21, 24 and 27 hours). Localisation of MII spindle on matured oocyte was examined by fluorescence staining using Hoechst 33345.

Maturation of LOPU-derived caprine oocytes were found to occur as early as 15 hours; however, the maturation rate (6.7%) was rather low. The maturation rate started to increase significantly ($P<0.05$) at 18 hours (53.3%) and peaked at 21 hours (86.7%). At 24 hours, the maturation rate was slightly reduced and a significant ($P<0.05$) drop in the maturation rate was observed at 27 hours (46.7%). Morphological evaluation on the PB-1 extrusion and its shape under the light microscope showed that more than 80% of the matured oocytes at 18 and 21 hours and 50% of the 24 hours matured oocytes had polar body with smooth surface (normal matured oocyte) and was not fully extruded (80 to 90%) from the cytoplast (Figure 4.2). However, at 27 hours, matured oocytes (>65%) had polar bodies that either fully extruded (100% separated from the cytoplast) or with fragmentation (abnormal oocyte) (Figure 4.3). In terms of the observation on the localisation of the MII spindle under the fluorescence microscope, matured oocytes at 24 hours started to show translocation of its MII spindle apart from the polar body; while MII spindle translocated even further apart from PB-1 in matured oocytes at 27 hours (Figure 4.4). In nuclear transfer experiment, the location of MII spindle close to PB-1 is important as it determines the success rate of enucleation using blind squeezing technique.

In contrast to LOPU-derived oocytes, commencement of maturation for abattoir-derived oocytes was found to occur later at 21 hours (53.3%). At 15 hours post-maturation, the abattoir derived oocytes were found to be in either metaphase I or anaphase I. Maturation rate of abattoir-derived oocytes peaked at 24 hours (80.0%) and slightly reduced ($P>0.05$) approaching 27 hours (73.3%). Majority of the matured oocytes (>80%) at 21 and 24 hours had polar body with smooth surface (normal matured oocyte) and was not fully extruded (80 to 90%) from the cytoplast. On the other hand, at 27 hours, some of the matured oocytes (<30%) had PB-1 that fully extruded (100% separated from the cytoplast) (Figure 4.5). Translocation of the MII spindle apart from PB-1 was observed only on matured oocytes at 27 hours (<30%).

Even though there were no significant differences ($P>0.05$) observed in the maturation rates at 21, 24 and 27 hours between the LOPU- and abattoir-derived oocytes, the quality of matured LOPU-derived oocytes in terms of the PB-1 morphology showed sign of fragmentation at 27 hours and translocation of the MII spindle apart from PB-1 started at 24 hours.

Table 4.5: Maturation rates (%, mean±SEM) of caprine oocytes retrieved from LOPU and abattoir at different IVM durations

Oocyte source	No. of replicates	Percentage of MII oocytes at different IVM durations				
		15 hours	18 hours	21 hours	24 hours	27 hours
LOPU	3	$6.7\pm6.7^{a,w}$ (1/15)	$53.3\pm6.7^{b,xy}$ (8/15)	$86.7\pm6.7^{a,z}$ (13/15)	$73.3\pm6.7^{a,yz}$ (11/15)	$46.7\pm6.7^{a,x}$ (8/15)
Abattoir	3	$0.0^{a,x}$ (0/15)	$0.0^{a,x}$ (0/15)	$53.3\pm17.6^{a,y}$ (9/15)	$80.0\pm11.5^{a,y}$ (12/15)	$73.3\pm13.3^{a,y}$ (11/15)

[ab]Means with different superscripts in a column within a group were significantly different ($P<0.05$).
[wxyz]Means with different superscripts in a row within a group were significantly different ($P<0.05$).

4.2.3 Evaluation on the Meiotic Competency of Caprine Oocyte Derived from Two Different Sources According to Oocyte Quality

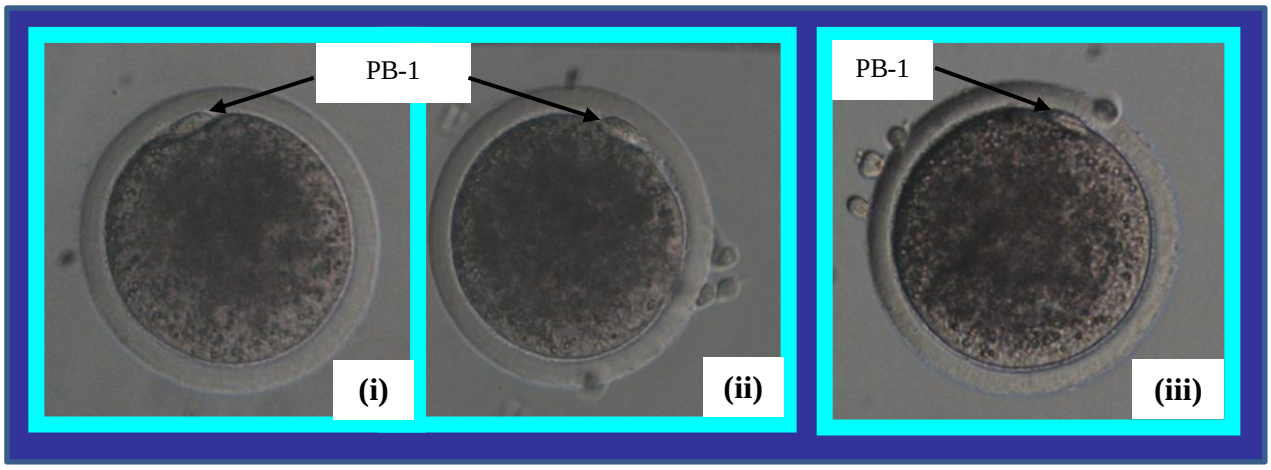
The maturation rates according to oocyte quality from two different sources, LOPU- versus abattoir-derived oocytes are shown in Table 4.6. The LOPU- and abattoir-derived oocytes were subjected to IVM duration of 18 to 22 hours and 22 to 26 hours, respectively, using the same formulation of IVM medium as depicted in Table 3.8. The overall maturation rate measured regardless of oocyte grades showed that LOPU-derived oocytes have significantly ($P<0.05$) higher maturation rate compared to the abattoir-derived oocytes (79.6% versus 69.7%). However, when the comparison was made within each oocyte grades, no significant differences ($P<0.05$) was observed in the maturation rate between LOPU- and abattoir-derived oocyte.

When considering the maturation rate of different oocyte grades in LOPU source, significantly ($P<0.05$) higher maturation rates were observed in oocytes of Grades A, B and C (percentage range: 61.1 to 84.4 %) compared to Grade D (28.6%). Similar trend was observed for the abattoir source in which the maturation rates of Grades A, B and C (percentage range: 73.1 to 79.6%) were significantly ($P<0.05$) higher compared to Grade D (45.2%).

Table 4.6: Maturation rate (%, mean±SEM) of caprine oocytes retrieved from LOPU- versus abattoir-derived ovaries according to oocyte quality using optimised IVM duration (LOPU: 18-22 hours; Abattoir: 22-26 hours)

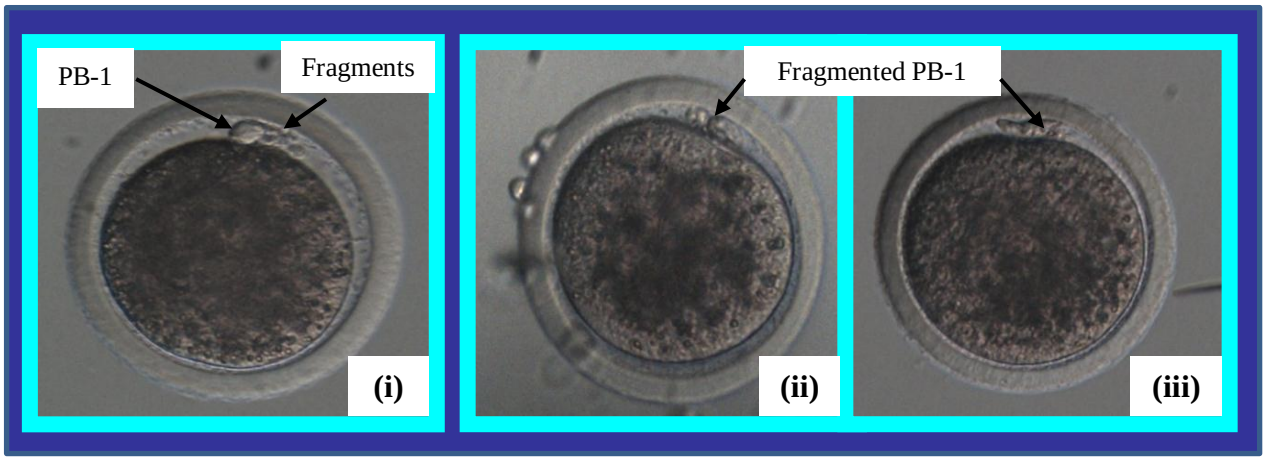
Oocyte source	No. of oocytes retrieved per doe	Maturation rate	Percentage of matured oocytes according to oocyte quality (n)			
			Grade A	Grade B	Grade C	Grade D
LOPU	11.0±0.86[a] (154/14)	79.6±2.6[b] (121/154)	84.4±3.3[a,y] (47/58)	82.1±4.9[a,y] (50/63)	61.1±11.6[a,y] (19/24)	28.6±11.4[a,x] (5/9)
Abattoir	20.2±1.2[b] (549/28)	69.7±3.7[a] (386/549)	76.4±4.2[a,y] (82/104)	79.6±3.1[a,y] (139/175)	73.1±5.7[a,y] (126/182)	45.2±7.5[a,x] (39/88)

[ab]Means with different superscripts in a column within a group were significantly different (P<0.05).
[xy]Means with different superscripts in a row within a group were significantly different (P<0.05).



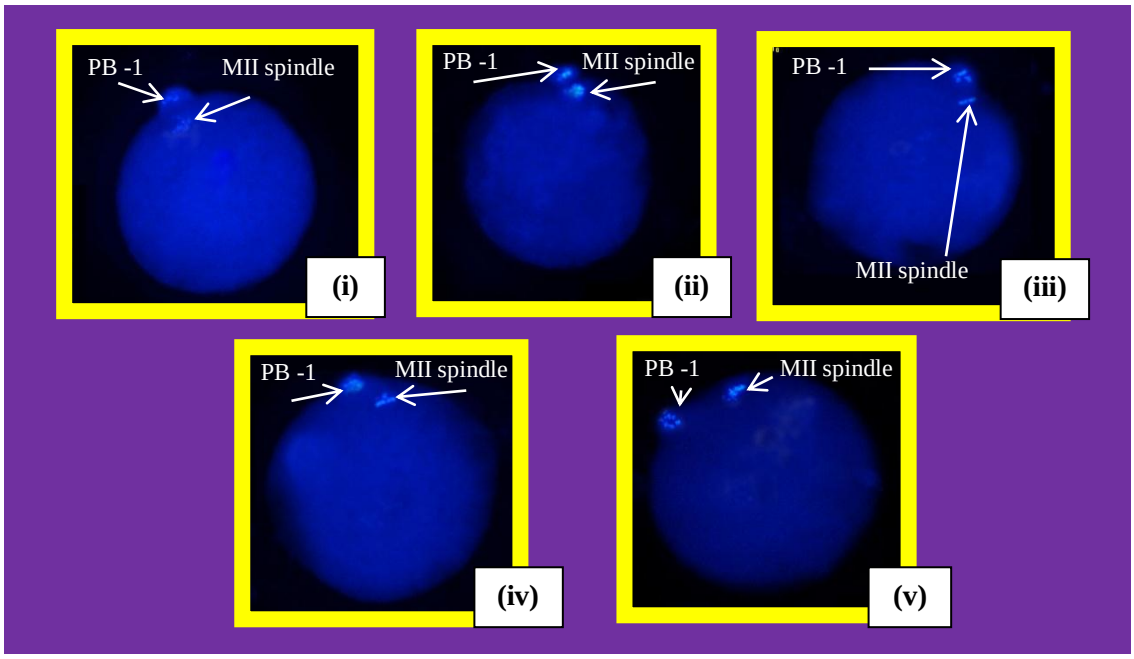
Note: (photomicrographs original magnification: 200x); (PB-1: first polar body)

Figure 4.2: Caprine *in vitro* matured oocytes with normal morphology of PB-1 (smooth surface and not fully extruded) obtained from:- (i-ii) LOPU oocytes at IVM duration 18-24 hours, (iii) abattoir oocyte at IVM duration 21-24 hours. Note: no difference was detected in oocyte morphology between LOPU and abattoir oocytes.



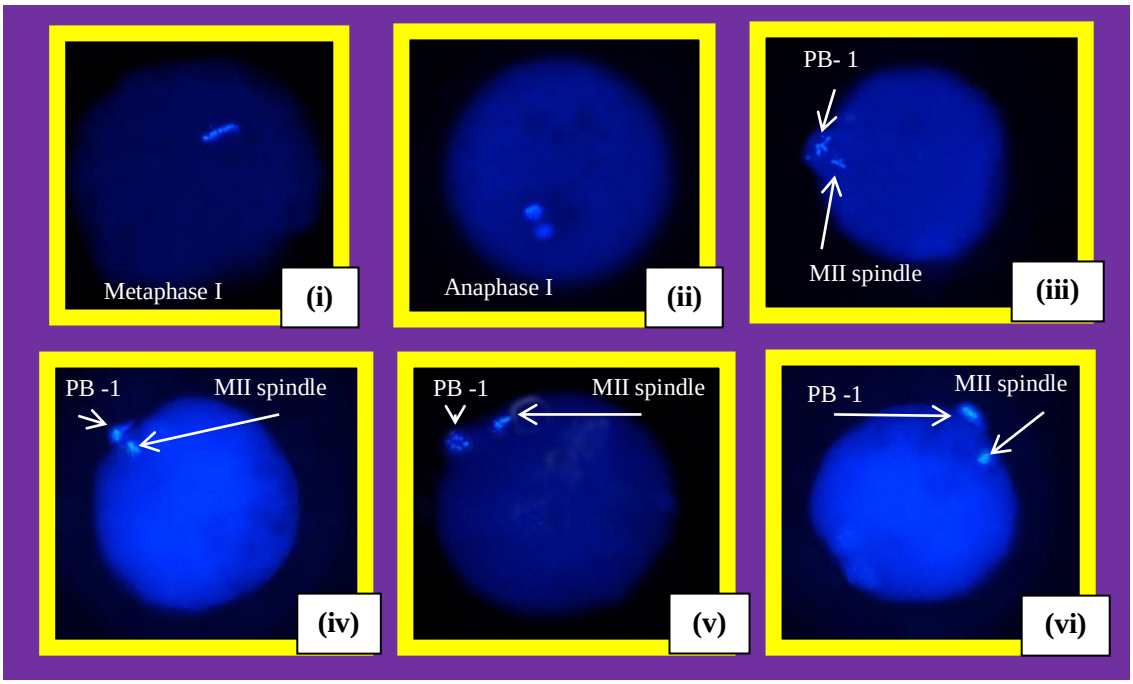
Note: (photomicrographs original magnification: 200x); (PB-1: first polar body)

Figure 4.3: Caprine *in vitro* matured oocytes with abnormal morphology of PB-1 (fragmented) obtained from:- (i) LOPU oocyte at IVM duration 27 hours, (ii-iii) abattoir oocytes at IVM duration 27 hours.



Note: (photomicrographs original magnification: 200x); (PB-1: first polar body); (MII: metaphase II)

Figure 4.4: LOPU-derived caprine oocyte *in vitro* matured at:- (i) 15 hours showing MII spindle located closely to PB-1, (ii) 18 hours showing MII spindle located closely to PB-1, (iii) 21 hours showing MII spindle located close to PB-1, (iv) 24 hours showing MII spindle start to translocate apart from PB-1 and (v) 27 hours showing MII spindle translocated far apart from PB-1.



Note: (photomicrographs original magnification: 200x); (PB-1: first polar body); (MII: metaphase II)

Figure 4.5: Abattoir-derived caprine oocyte at IVM duration of:- (i-ii) 15 hours showing oocyte at metaphase I and anaphase I, respectively; (iii) 18 hours showing MII spindle located closely to PB-1, (iv) 21 hours showing MII spindle located closely to PB-1, (v) 24 hours showing MII spindle translocated apart from PB-1 and (vi) 27 hours showing MII spindle translocated far apart from PB-1.

4.3 PRODUCTION OF CLONED BOVINE AND GAUR EMBRYOS VIA INTRASPECIES AND INTERSPECIES SCNT APPROACHES: A PRELIMINARY STUDY FOR CAPRINE SCNT RESEARCH (EXPERIMENT 3)

The manipulation efficiencies of bovine-bovine intraspecies SCNT (intraspSCNT) and gaur-bovine interspecies SCNT (interspSCNT) approaches are summarised in Table 4.7. There were a total of 181 and 203 matured bovine oocytes used in the bovine intraspSCNT and gaur interspSCNT studies, respectively. The success rate of manipulation in terms of enucleation and injection were high for both the bovine intraspSCNT (89.15% and 95.94%) and gaur interspSCNT (91.41% and 96.25%) in this preliminary attempt. Both bovine intraspSCNT and gaur interspSCNT approaches did not show any significant difference ($P>0.05$) in the fusion rate and cleavage rate. The morphology of matured bovine cumulus oocyte complexes (COCs) and the gaur ear fibroblast cell culture were presented in Figure 4.6.

The subsequent *in vitro* developmental (IVD) rates for both bovine intraspSCNT and gaur interspSCNT embryos are depicted in Table 4.8. There were a total of 93 out of 119 reconstructed bovine intraspSCNT oocytes developed to 2-cell stage giving an initial IVD percentage of 77.91%. The cloned bovine 2-cell embryo further developed to 4-cell, 8-cell, morula, compact morula, blastocyst and hatched blastocyst at the percentages of 69.85±2.48, 65.30±3.17, 46.12±2.42, 37.10±1.69, 20.29±2.59 and 18.63±2.14, respectively. There were significant reductions ($P<0.05$) in the IVD rate from 2-cell to 4-cell and 8-cell up to blastocyst stages. However, there was no significant reduction ($P>0.05$) in the percentage of cloned bovine blastocysts developed to hatched blastocysts.

As for the cloned gaur interspSCNT embryos, the IVD rate reduced non-significantly (P>0.05) from one stage to another starting with 2-cell (73.76±2.16), 4-cell (69.87±2.50), 8-cell (62.52±4.06) up to morula (45.86±1.36). However, IVD rate of cloned gaur embryos started to portray a significant reduction (P<0.05) in each embryo stage from morula (45.86±1.36), compact morula (34.46±2.14) up to blastocyst stage (20.91±3.90). The percentage of hatched blastocyst obtained was 19.02±3.35 in which the percentage of reduction did not differ significantly (P>0.05) from the blastocyst stage. When comparing the IVD rate between bovine intraspSCNT vs. gaur interspSCNT embryos across stages from 2-cell to hatched blastocyst, the IVD rates within each cell stage for both bovine intraspSCNT and gaur interspSCNT did not differ significantly (P>0.05). The morphology of *in vitro* developed gaur interspSCNT embryos were presented in Figure 4.7.

Table 4.7: Percentage (mean±SEM) of success in enucleation, injection, fusion and cleavage rate for bovine intraspSCNT and gaur interspSCNT embryos production

Type of SCNT approaches	No. of replicates	Percentage of oocytes successfully enucleated (n)	Percentage of oocytes successfully injected with donor karyoplast (n)	Percentage of couplets successfully fused* (n)	Percentage of reconstructed embryos cleaved* (n)
intraspSCNT (Bovine-bovine)	6	89.15±8.22 (162/181)	95.94±6.70 (157/162)	74.31±9.40[a] (119/157)	77.70±2.26[a] (93/119)
interspSCNT (Gaur-bovine)	7	91.41±6.34 (185/203)	96.25±5.15 (178/185)	70.43±7.68[a] (127/178)	73.75±5.71[a] (95/127)
Total	13	90.37 (347/388)	96.55 (335/347)	73.44 (246/335)	76.43 (188/246)

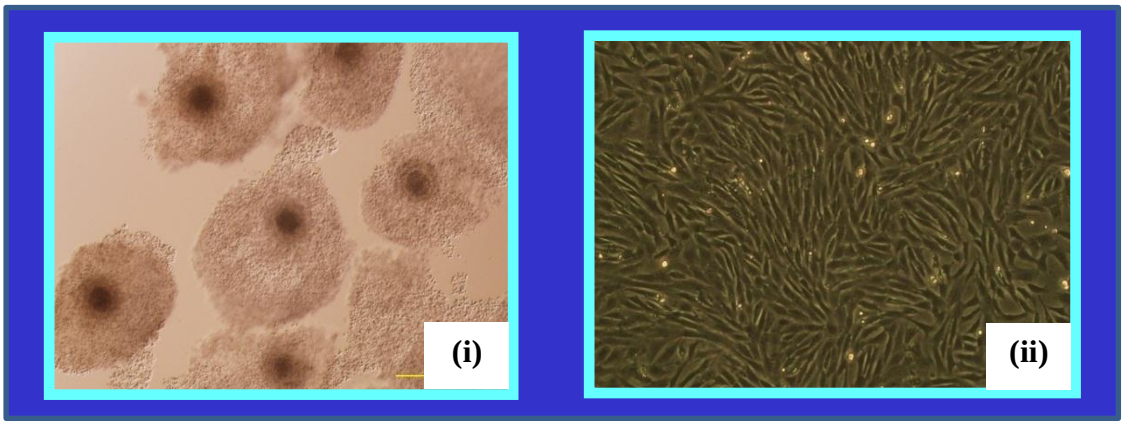
*Only fusion rate and cleavage rate were analysed with one-way ANOVA.

[a]Means with similar superscript in a column within a group were not significantly different (P>0.05).

Table 4.8: Percentage (mean±SEM) of *in vitro* developmental rate for bovine intraspSCNT and gaur interspSCNT cloned embryos

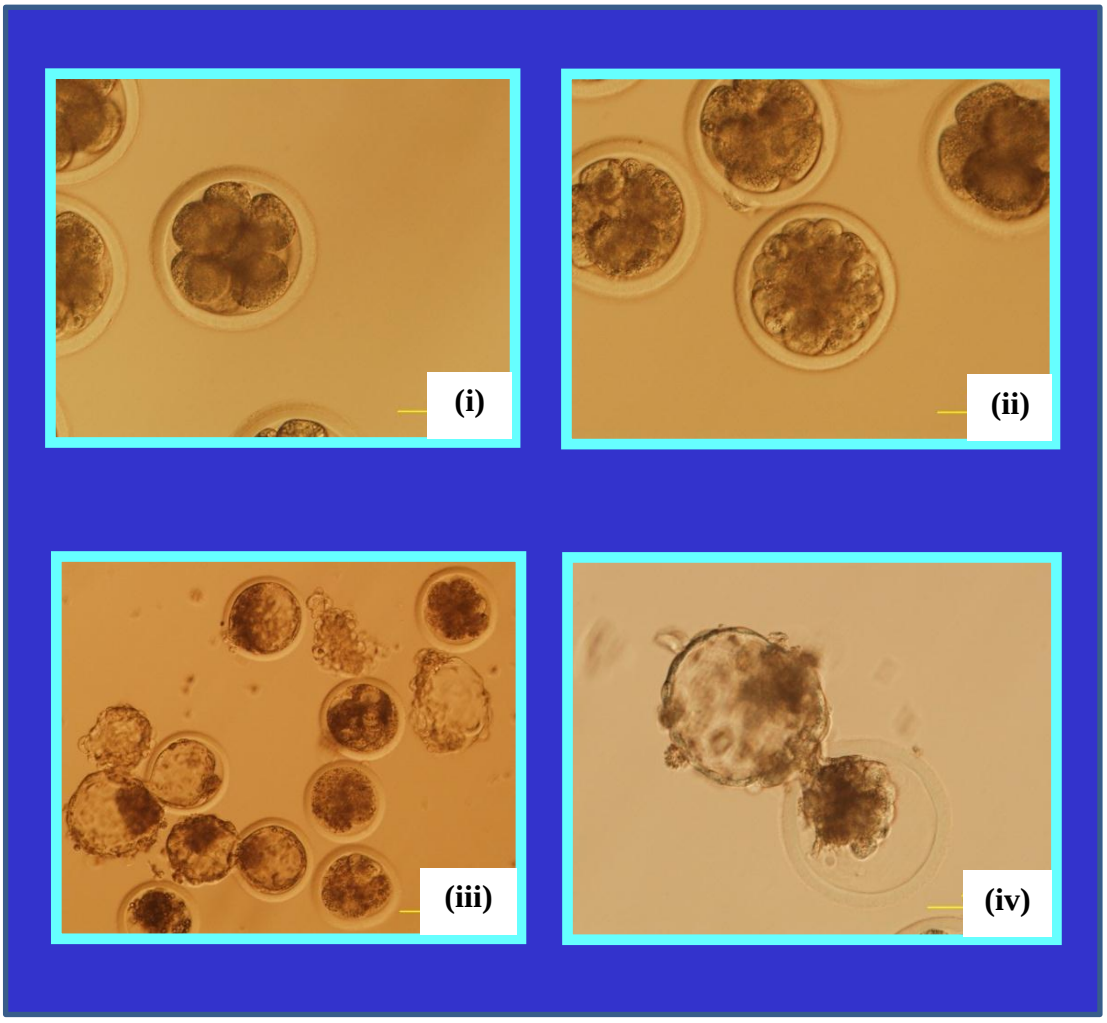
Type of SCNT approach	Percentage of cleaved reconstructed embryos according to cell stage (n)						
	2-cell	4-cell	8-cell	Morula	Compact morula	Blastocyst	Hatched blastocyst
intraspSCNT (Bovine-bovine)	77.91±0.93^{a,z} (93/119)	69.85±2.48^{a,y} (83/119)	65.30±3.17^{a,y} (77/119)	46.12±2.42^{a,x} (54/119)	37.10±1.69^{a,w} (43/119)	20.29±2.59^{a,v} (25/119)	18.63±2.14^{a,v} (23/119)
interspSCNT (Gaur- bovine)	73.76±2.16^{a,z} (95/127)	69.87±2.50^{a,yz} (90/127)	62.52±4.06^{a,xy} (79/127)	45.86±1.36^{a,x} (53/127)	34.46±2.14^{a,w} (45/127)	20.91±3.90^{a,v} (28/127)	19.02±3.35^{a,v} (25/127)
Total	75.67±1.33 (188/246)	69.86±1.69 (173/246)	63.81±2.56 (156/246)	45.98±1.27 (107/246)	35.68±1.39 (88/246)	20.62±2.32 (53/246)	18.84±1.98 (48/246)

^{a}Means with similar superscript in a column within a group were not significantly different (P>0.05).
^{vwwxyz}Means with different superscripts in a row within a group were significantly different (P<0.05).



Note: (photomicrographs original magnification: 40x)

Figure 4.6: (i) Abattoir-derived bovine COCs after maturation; (ii) Gaur ear fibroblast cell culture.



Note: (Original magnification of photomicrographs (i, ii, iv):100x; (iii) 40x)

Figure 4.7: *In vitro* development of gaur interspSCNT embryos. (i) 8-cell stage; (ii) compacting morula; (iii) early blastocyst and hatched blastocyst; (iv) hatched blastocyst.

4.4 IMPROVEMENT ON THE *IN VITRO* CLONED CAPRINE EMBRYOS PRODUCTION BY CONSIDERING THE EFFECTS OF MATURATION DURATION, ACTIVATION TREATMENT AND *IN VITRO* CULTURE PROTOCOL (EXPERIMENT 4)

The effect of three different factors, namely the maturation duration, activation treatment and *in vitro* culture (IVC) protocol on the *in vitro* developmental (IVD) rate of cloned caprine embryos were studied in this experiment. The best parameter that gave the highest IVD rate in each factor measured was used in the subsequent caprine nuclear transfer experiments.

4.4.1 Effect of Two Different IVM Intervals on Cloned Caprine IVD Competency using Ovarian- Superstimulated Caprine Oocyte

In this experiment, the maturation and manipulation rate as well as the IVD competency of cloned caprine embryos reconstructed using LOPU-derived oocyte matured at the IVM durations of 18 to 22 hours and 23 to 27 hours were evaluated. The maturation rate, percentage of successful enucleation, fusion rate and cleavage rate for oocytes matured in both IVM durations are shown in Table 4.9. LOPU-derived caprine oocytes matured at 18 to 22 hours (75.2%) showed significantly ($P<0.05$) higher maturation rate compared to oocytes matured at 22 to 27 hours (62.6%). In terms of the percentage of successful enucleation using blind squeezing technique, oocytes matured at 18 to 22 hours gave a significantly ($P<0.05$) higher success rate compared to oocytes matured at 23 to 27 hours (80.2% vs. 64.2%). No significant difference ($P>0.05$) was observed in the fusion rate between oocytes matured at 18 to 22 hours versus 23 to 27 hours. The

cleavage rate of the reconstructed oocytes matured at 18 to 22 hours was significantly higher ($P<0.05$) compared to oocyte matured at 23 to 27 hours (84.2% vs. 67.7%).

The reconstructed oocyte matured at 18 to 22 hours had significantly ($P<0.05$) higher IVD competency compared to reconstructed oocyte matured at 23 to 27 hours (Table 4.10). This was evident by the significantly ($P<0.05$) higher percentages of 2-cell, 8-cell and morula developed from reconstructed oocytes matured at 18 to 22 hours (84.2, 65.9, and 46.6%) versus oocyte matured at 23 to 27 hours (67.7, 46.4 and 24.7%). No blastocyst was obtained from the reconstructed oocytes matured in both the IVM durations tested.

Table 4.9: Percentage (%, mean±SEM) of maturation, enucleation, fusion and cleavage rate of LOPU derived caprine oocyte treated in 2 different IVM durations

IVM duration (hour)	Percentage of (n)			
	Maturation rate	Successful enucleation	Fusion rate	Cleavage rate
18-22	75.2±3.3[b] (63/83)	80.2±3.1[b] (50/63)	82.5±3.1[a] (41/50)	84.2±5.5[b] (34/41)
23-27	62.6±1.3[a] (68/109)	64.2±4.7[a] (44/68)	84.1±4.5[a] (37/44)	67.7±1.7[a] (25/37)

[ab]Means with different superscripts in a column within a group were significantly different ($P<0.05$).

Table 4.10: Percentage (%, mean±SEM) of *in vitro* developmental rate for cloned caprine embryos using LOPU derived oocytes matured in 2 different IVM intervals

IVM duration (hour)	Percentage of *in vitro* development (n)			
	2-cell	4-cell	8-cell	Morula
18-22	$84.2\pm5.5^{b,z}$ (34/41)	$74.7\pm4.1^{a,yz}$ (30/41)	$65.9\pm6.7^{b,y}$ (26/41)	$46.6\pm4.4^{b,x}$ (19/41)
23-27	$67.7\pm1.7^{a,z}$ (25/37)	$60.4\pm4.6^{a,z}$ (22/37)	$46.4\pm3.4^{a,y}$ (16/37)	$24.7\pm7.1^{a,x}$ (9/37)

[ab]Means with different superscripts in a column within a group were significantly different ($P<0.05$).
[xyz]Means with different superscripts in a row within a group were significantly different ($P<0.05$).

4.4.2 Effect of Two Different Activation Protocols on the *In Vitro* Developmental Competency of Reconstructed Caprine Embryos

The efficiency of two different type of sequential activation protocols on the IVD competency of reconstructed caprine embryos were evaluated in this experiment. A total of 67 successfully fused caprine oocytes were chemically activated using EtOH (7%) for 5 minutes followed with Cytochalasin D (1.25 µg/ml) and Cycloheximide (10 µg/ml) for 5 hours in the incubator (5% CO_2, 38.5°C); While another 59 fused caprine oocytes were chemically activated using Calcium Ionophore (5 µM) for 5 minutes followed with 6-Dimethylaminopurine (2 mM) for 4 hours in the incubator (5% CO_2, 38.5°C). The IVD competency of the reconstructed oocytes activated using the above mentioned activation protocols are depicted in Table 4.11.

No significant differences (P>0.05) observed between the IVD competency of reconstructed oocytes activated using 7% EtOH + CD-CHX versus CaI + 6-DMAP throughout the 2-cell (78.9% vs. 80.7%) to morula (40.0% vs. 42.8%) IVD stages. None of the activated reconstructed embryos cultured in this culture system using mSOFaa medium managed to develop beyond morula stage. Both activation protocol resulted in the similar decreasing *in vitro* development trend from 2-cell to morula.

Table 4.11: Percentage (%, mean±SEM) of *in vitro* developmental rate for reconstructed caprine embryos using two different sequential activation protocols

Type of activation protocol	No. of fused oocytes activated	Percentage of *in vitro* development (n)			
		2-cell	4-cell	8-cell	Morula
7% EtOH + CD-CHX	67	$78.9\pm1.3^{a,z}$ (53/67)	$68.8\pm0.8^{a,y}$ (46/67)	$58.0\pm2.3^{a,x}$ (39/67)	$40.0\pm1.2^{a,w}$ (27/67)
CaI + 6-DMAP	59	$80.7\pm2.9^{a,z}$ (48/59)	$70.8\pm1.8^{a,y}$ (42/59)	$57.4\pm1.5^{a,x}$ (34/59)	$42.8\pm2.7^{a,w}$ (25/59)

[a]Means with similar superscript in a column within a group were not significantly different (P>0.05).
[wxyz]Means with different superscripts in a row within a group were significantly different (P<0.05).

4.4.3 Effect of Two Different *In Vitro* Culture Media on the *In Vitro* Developmental Competency of Reconstructed Caprine Embryos

The IVD competency of reconstructed caprine embryos cultured in two *in vitro* culture (IVC) media namely, mSOFaa and KSOMaa (denoted as KSOMaa A in this experiment) were evaluated in this experiment (Table 4.12). A total of 58 and 70 reconstructed caprine oocytes activated with CaI (5 minutes) + 6-DMAP (4 hours) were cultured in mSOFaa and KSOMaa A respectively. Significant differences ($P<0.05$) were observed in the IVD competency between the reconstructed oocytes cultured in mSOFaa versus KSOMaa A at 2-cell (73.1% vs. 84.2%) and 8-cell (50.2% vs. 60.4%). However at 4-cell stage, no significant difference ($P>0.05$) was observed in the IVD rate of embryos cultured in mSOFaa and KSOMaa A. The KSOMaa A favourably supported the *in vitro* development of reconstructed embryos at later developmental stages in which the percentage of morula (42.8%) was significantly ($P<0.05$) higher compared to the embryos cultured in mSOFaa (32.1%) and the positive effect of KSOMaa A was even prominent when none of the reconstructed embryos cultured in mSOFaa managed to developed up to blastocyst stage compared to embryos cultured in KSOMaa A (3.85%). The developmental rates for the various stages of embryos showed similar descending trend for both the IVC media used.

Table 4.12: Percentage (%, mean±SEM) of *in vitro* developmental rate for cloned caprine embryos cultured in mSOFaa versus KSOMaa

Type of IVC medium	No of reconstructed oocyte cultured	Percentage of *in vitro* development (n)					
		2-cell	4-cell	8-cell	Morula	Blastocyst	Hatched blastocyst
mSOFaa	58	$73.1\pm2.3^{a,z}$ (42/58)	$62.9\pm6.1^{a,z}$ (35/58)	$50.2\pm2.7^{a,y}$ (29/58)	$32.1\pm2.8^{a,x}$ (18/58)	$0.0\pm0.0^{a,w}$ (0/58)	0.0 (0/58)
KSOMaa A	70	$84.2\pm3.2^{b,z}$ (59/70)	$71.5\pm3.9^{a,y}$ (50/70)	$60.4\pm3.1^{b,x}$ (42/70)	$42.8\pm2.7^{b,w}$ (30/70)	$3.85\pm2.3^{a,v}$ (3/70)	0.0 (0/70)

[ab]Means with different superscripts in a column within a group were significantly different ($P<0.05$).
[vwxyz]Means with different superscripts in a row within a group were significantly different ($P<0.05$).

4.4.4 Effect of Increasing Glucose Concentration in KSOMaa medium at Day 2 culture on the *In Vitro* Developmental Competency of Reconstructed Caprine Embryos

The effect of increasing glucose in KSOMaa B to the final concentration of 2.78 mM for Day- 2 to 8 (Treatment B IVC) versus the used of classic KSOMaa A formulation throughout the 8 days (Treatment A IVC) on the IVD competency of cloned caprine embryos were evaluated (Table 4.13). The IVD competency of reconstructed caprine embryos for early developmental stages (2- to 8-cell) cultured in Treatment A IVC versus Treatment B IVC did not differ significantly ($P>0.05$). However, the effect of increasing glucose concentration in Treatment B IVC increased the morula (46.7%), blastocyst (19.9%) and hatched blastocyst (15.6%) rates of the reconstructed caprine embryos significantly ($P<0.05$) compared to caprine embryos cultured in Treatment A IVC. None of the cloned caprine blastocyst cultured in Treatment A IVC managed to hatch.

Table 4.13: Percentage (%, mean±SEM) of *in vitro* developmental rate for cloned caprine embryos cultured in two different IVC treatments

Type of IVC treatment	No of reconstructed oocyte cultured	Percentage of *in vitro* development (n)					
		2-cell	4-cell	8-cell	Morula	Blastocyst	Hatched blastocyst
Treatment A	122	$76.8\pm2.2^{a,z}$ (94/122)	$68.1\pm1.7^{a,y}$ (83/122)	$58.2\pm2.3^{a,x}$ (71/122)	$34.9\pm2.3^{a,w}$ (42/122)	$3.8\pm1.5^{a,v}$ (5/122)	$0.0\pm0.0^{a,v}$ (0/122)
Treatment B	131	$77.4\pm1.9^{a,z}$ (103/131)	$68.5\pm2.6^{a,y}$ (91/131)	$60.4\pm1.6^{a,x}$ (80/131)	$46.7\pm1.7^{b,w}$ (61/131)	$19.9\pm1.5^{b,v}$ (26/131)	$15.6\pm2.5^{b,v}$ (18/131)

^{ab}Means with different superscripts in a column within a group were significantly different ($P<0.05$).
^{vwxyz}Means with different superscripts in a row within a group were significantly different ($P<0.05$).

Note: Treatment A IVC: IVC in KSOMaa for 8 days;
Treatment B IVC: IVC in KSOMaa for 2 days + KSOMaa with final concentration of 2.78 mM glucose for another 6 days.

4.5 EFFICACY OF PRODUCING CLONED CAPRINE EMBRYOS USING INTRASPECIES VERSUS INTERSPECIES SCNT APPROACHES (EXPERIMENT 5)

The efficacy of two different SCNT approaches, namely the intraspecies SCNT (intraspSCNT) and interspecies SCNT (interspSCNT) on the *in vitro* developmental (IVD) rate of cloned caprine embryos were evaluated in this experiment. The quality of the cloned caprine hatched blastocysts generated using both approaches were evaluated by enumerating the embryonic cell number after fluorescence staining. Embryo transfer (ET) attempts were also conducted to test the implantation ability of both the intraspSCNT and interspSCNT caprine embryos.

The *in vitro* development of the cloned caprine embryos using intraspSCNT approach was compared with the *in vitro* development of caprine parthenotes that served as a control group (Table 4.14). A total of 102 successfully fused caprine intraspSCNT embryos and 70 activated caprine oocytes were cultured in IVC medium Treatment B. The *in vitro* development of caprine intraspSCNT and parthenotes were observed from Day 2 onwards to Day 8. The results obtained in this experiment showed that the IVD rate of caprine intraspSCNT embryos at 2-cell and 4-cell stage did not differ significantly from the caprine parthenotes. However, the IVD competency of caprine intraspSCNT embryos were significantly ($P<0.05$) higher at 8-cell (61.2%) and morula (47.6%) stages compared to caprine parthenotes (55% and 35.3%, respectively). When approaching blastocyst stage and hatched blastocyst, the IVD competency of caprine intraspSCNT embryos (17.3% and 11.3% respectively) and parthenotes (15% and 9.0% respectively) were similar ($P>0.05$).

Table 4.14: Percentage (%, mean±SEM) of *in vitro* developmental rate for caprine intraspSCNT and parthenogenesis embryos

Type embryos	Percentage of cleaved reconstructed caprine intraspSCNT and PA embryos according to cell stage (n)					
	2-cell	4-cell	8-cell	Morula	Blastocyst	Hatched blastocyst
intraspSCNT caprine	$78.09\pm2.7^{a,z}$ (81/102)	$67.97\pm3.33^{a,y}$ (71/102)	$61.22\pm2.17^{b,x}$ (63/102)	$47.60\pm2.07^{b,w}$ (48/102)	$17.31\pm1.83^{b,v}$ (19/102)	$11.27\pm1.02^{b,v}$ (11/102)
PA caprine	$74.10\pm2.72^{a,z}$ (50/70)	$65.33\pm3.89^{a,y}$ (44/70)	$55.00\pm1.29^{a,x}$ (38/70)	$35.33\pm2.00^{a,w}$ (24/70)	$15.00\pm1.29^{b,v}$ (11/70)	$9.00\pm2.77^{b,v}$ (5/70)

^{ab}Means with different superscripts in a column within a group were significantly different ($P<0.05$).
^{vwxyz}Means with different superscripts in a row within a group were significantly different ($P<0.05$).

In a separate experiment, cloned caprine embryos were generated using interspSCNT approach in which, the bovine oocytes were used as the recipient cytoplasts and the caprine ear fibroblast cells were used as the donor karyoplasts. The IVD competency of caprine interspSCNT embryo was evaluated and comparison in the IVD competency was made with the IVD competency of bovine parthenotes (Table 4.15).

A total of 165 reconstructed bovine oocytes using interspSCNT approach were cultured in Treatment B IVC; while for the bovine parthenotes, a total of 115 bovine oocytes were activated and subjected to the same culture system. The IVD competency at 2-cell stage for caprine interspSCNT (78%) embryos did not differ significantly ($P>0.05$) from bovine parthenotes (73.5%). However, at 4-cell and 8-cell, the IVD rates of bovine parthenotes (63.6% and 56.7%, respectively) was significantly ($P<0.05$) lower compared to the caprine interspSCNT embryos (74.3% and 63.2%, respectively). When approaching morula, blastocyst and hatched blastocyst stage, the IVD competency of caprine interspSCNT embryos (32.8%, 8.54% and 4.82%, respectively) did not differ significantly ($P>0.05$) from the bovine parthenotes (36.1%, 12.6% and 7.9%, respectively).

Table 4.15: Percentage (%, mean±SEM) of *in vitro* developmental rate for caprine interspSCNT and bovine parthenogenesis embryos

Type of embryos	Percentage of cleaved reconstructed caprine interspSCNT and PA embryos according to cell stage (n)					
	2-cell	4-cell	8-cell	Morula	Blastocyst	Hatched blastocyst
interspSCNT caprine	$78.03\pm1.00^{a,z}$ (129/165)	$74.28\pm1.66^{b,y}$ (122/165)	$63.16\pm1.32^{b,x}$ (104/165)	$32.75\pm1.79^{a,w}$ (54/165)	$8.54\pm0.60^{b,v}$ (14/165)	$4.82\pm0.59^{b,u}$ (8/165)
PA bovine	$73.45\pm2.45^{a,z}$ (84/115)	$63.56\pm2.78^{a,y}$ (73/115)	$56.72\pm1.52^{a,x}$ (65/115)	$36.06\pm1.37^{a,w}$ (41/115)	$12.56\pm1.67^{b,v}$ (14/115)	$7.89\pm1.79^{b,v}$ (10/115)

^{ab}Means with different superscripts in a column within a group were significantly different ($P<0.05$).
^{uvwxyz}Means with different superscripts in a row within a group were significantly different ($P<0.05$).

The efficacy between the intraspSCNT and interspSCNT approaches was evaluated in terms of the success in the manipulation procedure and the IVD competency. The percentage of caprine and bovine oocyte maturation, the success in enucleation, fusion and cleavage rate between the intraspSCNT and interspSCNT approaches are tabulated in Table 4.16. The maturation rate between caprine (79.7%) and bovine (78.1%) oocytes using the same IVM medium did not differ significantly ($P>0.05$). The percentage of oocytes successfully enucleated and fused for both matured caprine oocyte for intraspSCNT (90.9% and 82.6%) and bovine oocyte for interspSCNT (88.1% and 75.3%) did not differ significantly ($P\geq0.05$) as well.

Both caprine intraspSCNT and interspSCNT reconstructed embryos also showed similar ($P>0.05$) cleavage rates (78.1% and 78.0%, respectively). The caprine interspSCNT approach gave significantly ($P<0.05$) higher 4-cell embryos compared to intraspSCNT approach (Table 4.17). However, the IVD competency reaching 8-cell stage did not differ significantly ($P>0.05$) between both approaches. The caprine intraspSCNT embryos were able to develop more favourably ($P<0.05$) compared to interspSCNT embryos at morula (47.6% and 32.8%, respectively), blastocyst (17.3% and 8.5%, respectively) and hatched blastocyst (11.3% and 4.8%, respectively).

Table 4.16: Percentage (%, mean±SEM) of success in enucleation, injection, fusion and cleavage rate for caprine intraspSCNT and interspSCNT embryos

Type of SCNT approaches	Percentage of maturation (n)	Percentage of oocytes successfully enucleated (n)	Percentage of couplets successfully fused (n)	Percentage of reconstructed embryos cleaved (n)
intraspSCNT (Caprine-caprine)	79.7±2.9[a] (135/165)	90.9±2.5[a] (121/135)	82.6±3.4[b] (102/121)	78.1±2.7[a] (81/102)
interspSCNT (Caprine-bovine)	78.1±1.9[a] (248/319)	88.1±2.1[a] (219/248)	75.3±0.9[a] (165/219)	78.0±1.0[a] (129/165)
Total	78.8±1.6 (383/484)	89.3±1.6 (340/383)	78.6±1.9 (267/340)	78.1±1.3 (210/267)

[ab]Means with different superscript in a column within a group were significantly different ($P \leq 0.05$).

Table 4.17: Percentage (%, mean±SEM) of *in vitro* developmental rate for caprine intraspSCNT and interspSCNT embryos

Type of NT approaches	Percentage of cleaved reconstructed caprine intraspSCNT and interspSCNT embryos according to cell stage (n)					
	2-cell	4-cell	8-cell	Morula	Blastocyst	Hatched blastocyst
intraspSCNT caprine	78.09±2.7^{a,z} (81/102)	67.97±3.33^{a,y} (71/102)	61.22±2.17^{a,x} (63/102)	47.60±2.07^{b,w} (48/102)	17.31±1.83^{b,v} (19/102)	11.27±1.02^{b,v} (11/102)
interspSCNT caprine	78.03±1.00^{a,z} (129/165)	74.28±1.66^{b,y} (122/165)	63.16±1.32^{a,x} (104/165)	32.75±1.79^{a,w} (54/165)	8.54±0.60^{a,v} (14/165)	4.82±0.59^{a,u} (8/165)

^{ab}Means with different superscripts in a column within a group were significantly different (P<0.05).
^{uvwxyz}Means with different superscripts in a row within a group were significantly different (P<0.05).

The assessment on the embryonic cell number of caprine intraspSCNT, interspSCNT and parthenotes at hatched blastocyst stage are shown in Table 4.18. The mean cell number of caprine hatched blastocysts derived from interspSCNT (81.4) was significantly lower ($P<0.05$) compared to intraspSCNT (109), caprine parthenogenesis (107.3) and bovine parthenogenesis (112.2) hatched blastocysts. There was no significant difference between cell numbers of caprine intraspSCNT and caprine parthenogenesis hatched blastocyst.

Table 4.18: Cell number (mean±SEM) of cloned caprine and PA hatched blastocysts

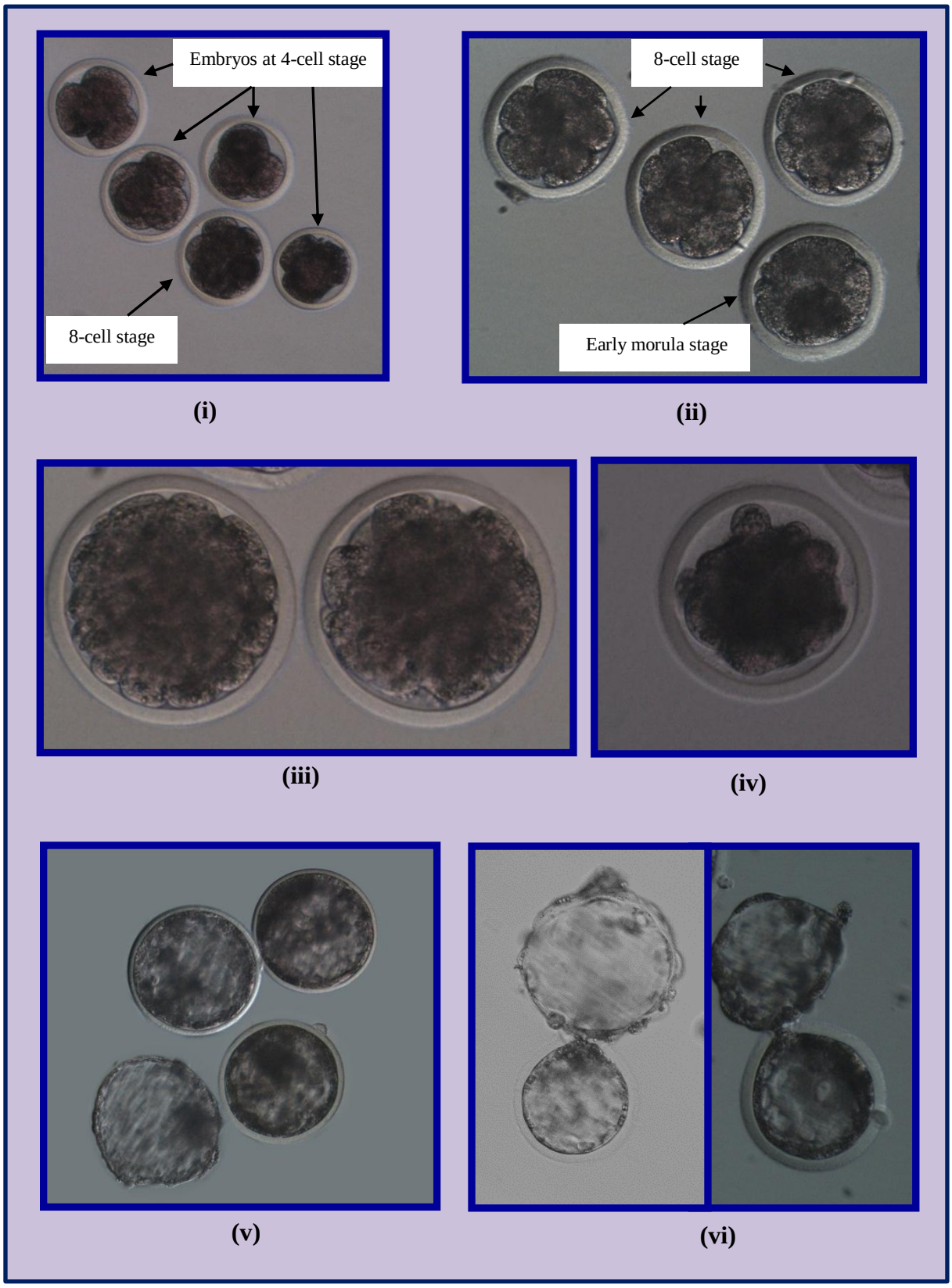
Type of embryo	Number of hatched blastocyst stained	Cell number ± SEM
Caprine intraspSCNT	5	109 ± 4.7^{b}
Caprine interspSCNT	5	81.4 ± 2.6^{a}
Caprine PA	4	107.3 ± 5.1^{b}
Bovine PA	5	112.2 ± 4.6^{b}

[ab]Means with different superscripts in a column within a group were significantly different ($P<0.05$).

Eight ET attempts were conducted using two approaches, namely oviduct transfer and uterine transfer (Table 4.19). A total of 26 caprine intraspSCNT embryos (2-cell, 4-cell, 8-cell and morula) were transferred together with 4 caprine parthenotes (8-cell) into 5 recipients with the presence of at least 1 corpus luteum (CL). Another 3 recipients with the presence of at least 1 CL were transferred with 12 caprine interspSCNT embryos (4-cell, 8-cell and morula). Ultrasound scanning conducted on day 30 post-ET revealed that there were no pregnancy detected on all the eight recipient does.

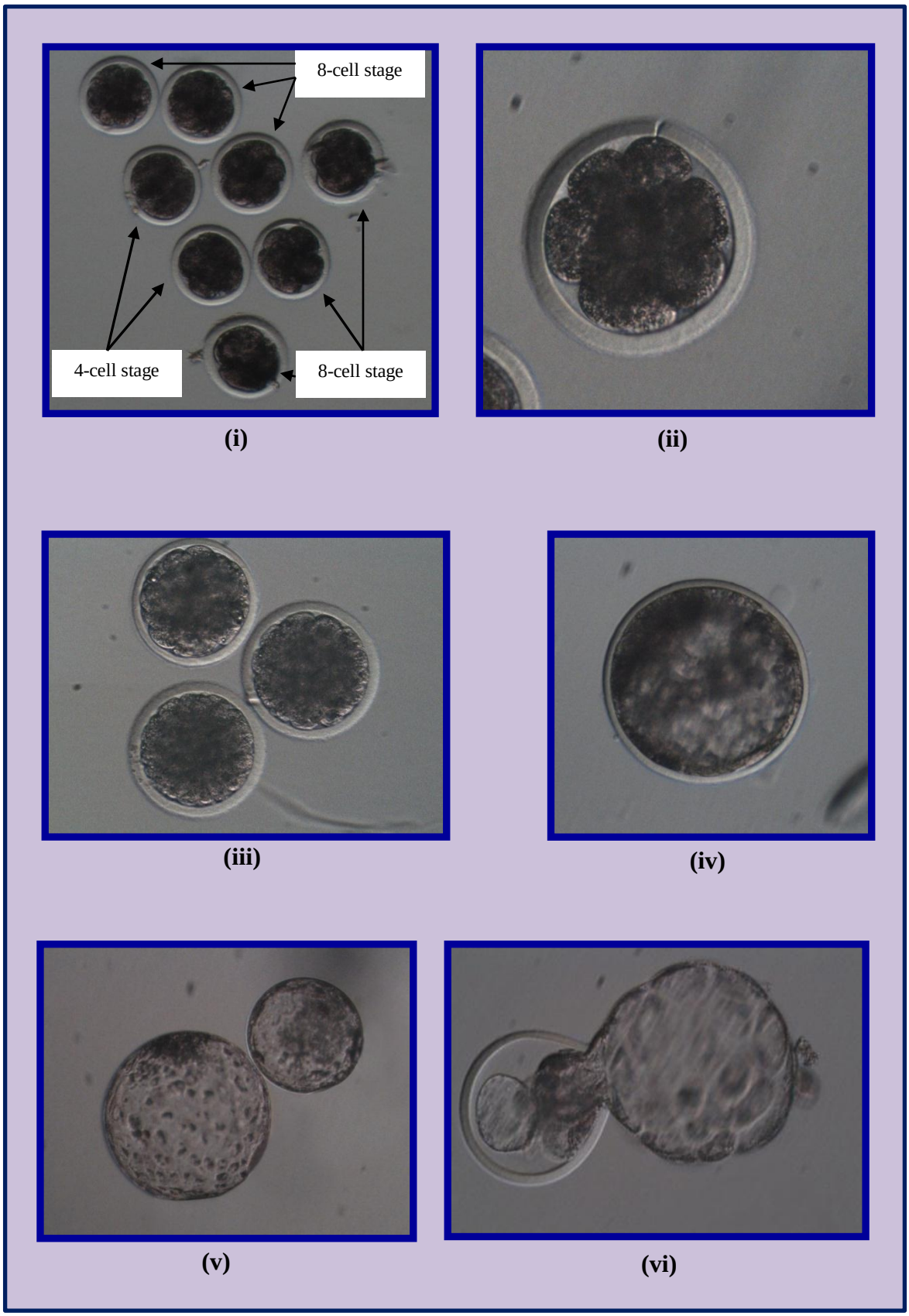
Table 4.19: Embryo transfer attempts of intraspSCNT and interspSCNT cloned caprine embryos

No. of Rep	Recipient ID synchronised	No. of corpus luteum		Type of caprine embryo transferred	Type of ET approach	No. of embryos transferred (embryo stages)		Pregnancy rate (detection 30 Days post-ET via ultrasonography)
		Left ovary	Right ovary			Left	Right	
1	0019	1	2	intraspSCNT	Oviduct transfer	1 (2-cell), 1 (4-cell)	1 (2-cell), 1 (4-cell)	0
	0014	2	3	intraspSCNT	Oviduct transfer	3 (4-cell)	2 (4-cell), 1 (8-cell)	0
2	0112	2	1	intraspSCNT + PA	Oviduct transfer	4 SCNT (8-cell), 2 PA (8-cell)	4 SCNT (8-cell), 2 PA (8-cell)	0
	0070	0	0	-	-	-	-	-
3	0068	0	1	intraspSCNT	Uterine transfer	1 (4-cell), 1 (8-cell)	2 (8-cell)	0
	B0126	0	2	intraspSCNT	Uterine transfer	2 (morula)	2 (morula)	0
	B0101	0	0	-	-	-	-	-
4	0007	0	0	-	-	-	-	-
	0216	1	1	interspSCNT	Oviduct transfer	1 (4-cell), 1 (8-cell)	1 (8-cell), 1 (morula)	0
	B0114	1	0	interspSCNT	Uterine transfer	1 (8-cell), 1 (morula)	2 (8-cell)	0
5	8675	0	3	interspSCNT	Uterine transfer	2 (8-cell)	2 (morula)	0
	B0163	0	0	-	-	-	-	-
TOTAL		7	13			19 (NT embryos), 2 (PA embryos)	19 (NT embryos), 2 (PA embryos)	0



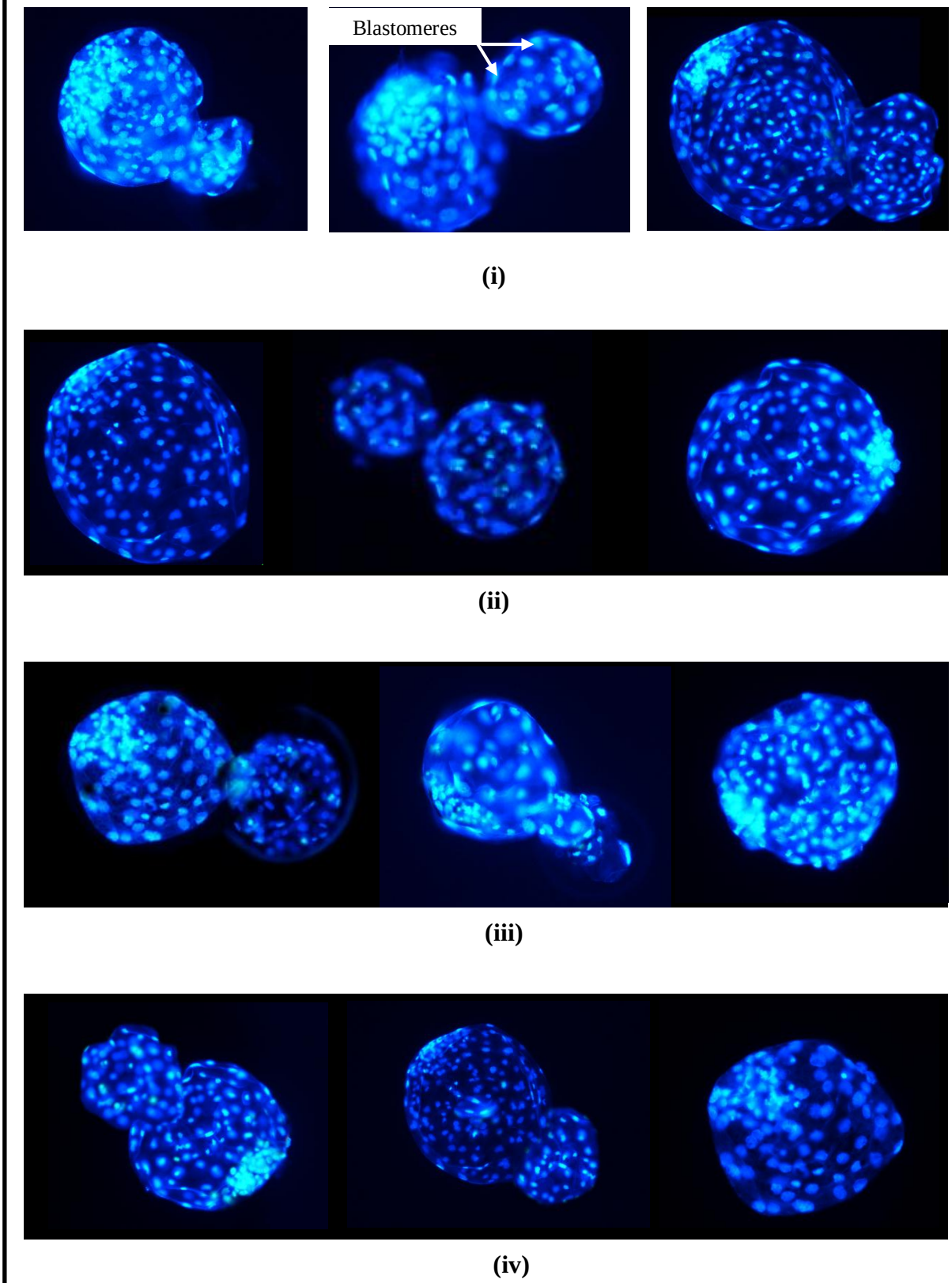
Note: (Original magnification of photomicrographs (i): 40x; (ii, v, vi):100x; (iii, iv) 200x)

Figure 4.8: *In vitro* development of caprine intraspSCNT embryos. (i) four embryos at 4-cell stage; (ii) three embryos at 8-cell stage and one early morula; (iii) two embryos at morula stage; (iv) compacted morulae; (v) four blastocysts; (vi) two hatched blastocyst.



Note: (Original magnification of photomicrographs (i): 40x; (iii, v): 100x; (ii, iv, vi): 200x)

Figure 4.9: *In vitro* development of caprine interspSCNT embryos. (i) two embryos at 4-cell stage and six embryos at 8-cell stage; (ii) 8-cell stage embryo; (iii) three morula; (iv) early blastocyst; (v) two blastocysts; (vi) hatched blastocyst.



Note: (Original magnification of photomicrographs (i-iv): 200x)

Figure 4.10: Fluorescent staining of hatched blastocysts derived from; (i) caprine intraspSCNT, (ii) caprine interspSCNT, (iii) caprine PA, (iv) bovine PA.

Chapter 5

5.0 DISCUSSION

Chapter 5

5.0 DISCUSSION

5.1 EFFECT OF DIFFERENT SOURCES OF GONADOTROPHIN ON CAPRINE SUPERSTIMULATORY RESPONSES (EXPERIMENT 1)

It is important to select a suitable source(s) of gonadotrophin to superstimulate the donor goats in order to maximise the quantity and quality of caprine oocyte yield per LOPU session. The previous studies (Phua, 2006; Anna, 2007; Rahman, 2008) pertaining to caprine superstimulatory response in the present laboratory routinely used oFSH (Ovagen®) together with hCG (Ovidrel®) treatment. However, due to some technical problem at manufacture level, oFSH (Ovagen®) was temporarily discontinued from production. As an alternative to replace oFSH (Ovagen®), two different sources of gonadotrophin, namely PMSG (Folligon®) and pFSH (Folltropin®-V) were employed in this study, and the efficacy of these sources of gonadotrophin on caprine ovarian responses for 3 OR cycles were evaluated.

Generally, the superstimulatory effect of PMSG (Table 4.1) in terms of the average number of follicles recruited and the quantity of oocytes retrieved in this study (16.4 and 11, respectively) were comparable to the results obtained by Kong (2010) in her study (17.7 and 10.1, respectively) on the effect of PMSG using 1200 IU and 1500 IU dosages in caprine for 2 OR cycles. The present study also obtained similar findings with Kong (2010), in which no significant differences ($P>0.05$) were observed in the average numbers of follicles recruited and the quantity of oocytes retrieved between OR1 and OR2. The number of oocyte retrieved in OR2 slightly increased compared to OR1, and this might be due to the effect of increased dosage of PMSG (1500 IU).

administered in OR2. However, when approaching OR3, the number of follicles recruited (9.7) and the quantity of oocytes retrieved (6.3) decreased significantly ($P<0.05$) compared to the previous OR cycle. This indicated that the level of caprine ovarian response towards PMSG (1500 IU) was not greatly influence as in OR2 at the third repeated gonadotrophin stimulation and LOPU cycles. Similar decreasing effect of repeated ovarian stimulation using PMSG and hCG in mouse were observed and was reported by Combelles *et al*. (2003) in their study; fewer oocytes were retrieved from female mice at the fourth consecutive cycle of PMSG and hCG stimulation.

However, according to Anel *et al*. (1997) and Stangl *et al*. (1999), repeated gonadotrophin stimulation using PMSG (1500 IU) and LOPU cycles (6 and 20 cycles, respectively) had no negative influence on the ovine follicular response and oocyte yield. This contradictory outcome might be due to the additional usage of monoclonal antibodies against PMSG (antiPMSG) in their stimulation regime which was not employed in our present study. Repeated use of PMSG has been reported to result in poor fertility in goats (Chemineau *et al*., 1999). These reasons have been attributed to the presence of anti-PMSG antibodies developed as an immune response to previous gonadotrophin treatments (Baril *et al*., 1995). The presence of such antibody was reported to cause a delay in the occurrence of oestrus, LH peak and ovulation in the synchronised does, which may explain the lower fertility status (Bavister *et al*., 1986; Chemineau *et al*., 1999; Roy *et al*., 1999; Drion *et al*., 2001). In conjunction to this study, perhaps the caprine ovarian response decreased by OR3 was due the presence of accumulative anti-PMSG antibodies provoked from the previous gonadotrophin stimulation treatments in OR1 and 2. Nonetheless, there was possibility that the results obtained in OR3 might also be affected by the existence of variables in the hormonal responsiveness that occurred between animals and replicates as well as the different batches of hormone used as reported by Cognié *et al*. (2004).

Even though the superstimulatory effect of PMSG decreased after 3 repeated OR cycles, the average number of oocytes retrieved (11) per doe for the overall 3 OR cycles was relatively higher compared to the studies conducted by Rosnina *et al*. (1992) on goats (3 oocytes per doe), Anel *et al*. (1997) and Stangl *et al*. (1999), who obtained 7 and 7.6 oocytes per ewe, respectively, from repeatedly stimulated ewe using PMSG/antiPMSG regime. The effect of different stimulation regime and species difference might be the factors contributed to the variation in the overall average numbers of oocytes retrieved per doe in this study.

The repeated cycles of superstimulation using PMSG and LOPU did not affect the quality of oocytes yielded as oocytes of Grades A, B and C which were found to be the majority in all the 3 OR cycles. Since the oocyte quality was not compromised in this repeated superstimulation and OR cycle tested, the application of PMSG as gonadotrophin to superstimulate donor does can be considered as well as improvement on the oocyte yield when the OR cycle increases could be carried out in future by incorporating the use of antiPMSG at the appropriate dosage.

The effect of repeated superstimulation using pFSH to the caprine ovarian response was evaluated (Table 4.2). No significant differences were observed in the number of oocytes retrieved per ovary among the 3 OR cycles. However, the number of oocytes retrieved in the first OR was the highest and this number steadily declined for the consecutive OR cycles. Similar finding was reported by Baldassarree *et al*. (2003) who repeatedly stimulated the donor does with pFSH coupled with eCG. Even though the stimulation regime in this study was not coupled with eCG administration, instead substitution was made using hCG, the overall caprine follicles and oocytes yielded from does stimulated for the first cycle (16.4 and 12.5, respectively) and the third repeated cycle (14 and 11.5, respectively) were comparable to the results reported by

Baldassarree *et al*. (2003) for OR1 (17.9 follicles and 16.3 oocytes) and OR3 (11.1 follicles and 8.8 oocytes) cycles. Armstrong *et al*. (1992, 1994) reported that pFSH without accompanying with PMSG was ineffective in stimulating cattle follicle development, however, when coupled with hCG treatment, follicle development was enhanced and oocytes recovered were competent to undergo maturation *in vitro*.

The overall average number of oocytes yielded from ovaries stimulated with pFSH in this study (12.1 oocytes per doe) was relatively higher compared to the study conducted by Rosnina *et al*. (1992) who stimulated does using multiple injection of 10 to 20 mg of FSH without coupling with hCG (6.8 oocytes per doe). The relatively higher oocyte yield obtained in this study showed that the stimulation regime using higher dosage of pFSH (70 mg) coupled with hCG was more efficient compared to the goat stimulation regime used by Rosnina *et al*. (1992).

The steadily and non-significant declining trend in the number of oocytes retrieved in the 3 consecutive OR cycles studied might be due to the effect of repeated follicular puncture which could alter the endocrine profiles slightly, and thus, cause minor morphological changes in the ovaries (Petyim *et al*., 2001). Generally, the oocytes yielded from does stimulated with pFSH for all the 3 OR cycles was of good quality as the distribution of oocytes graded were majority of Grades A and B categories. No significant differences were observed in the number of oocytes yielded within each grade for the 3 OR cycles. In other words, the quality of oocytes yielded via LOPU was not compromised, even though the donor does were subjected to 3 repeated stimulation cycles using pFSH. These findings are consistent and in agreement with Alberio *et al*. (2002), Baldassarre *et al*. (2003) and Gibbons *et al*. (2007) that good quality oocytes remained unaffected between OR cycles.

The effect of PMSG vs. pFSH stimulation on the average number and quality of oocytes retrieved in each OR cycle were evaluated (Table 4.3). There were no significant differences in the number of follicles recruited and number of oocytes retrieved from does stimulated with PMSG vs. pFSH in OR1 and OR2. However, in OR3, the caprine ovary stimulation response using PMSG was significantly lower compared to pFSH in which the number of oocytes yielded from PMSG stimulated ovaries were significantly lower compared to pFSH stimulated ovaries. In terms of oocyte quality, the pFSH stimulated ovaries were likely to produce slightly better oocyte quality than PMSG stimulated ovaries. This is in conjunction with the observation that generally the percentages of Grades A and B oocyte yield from pFSH stimulated ovaries were relatively higher than PMSG stimulated ovaries.

The overall findings in this experiment revealed that repeated ovarian stimulation using PMSG was not as efficient as using pFSH which is in agreement with the findings reported by Nuti *et al*. (1987), Mahmood *et al*. (1991) and Nowshari *et al*. (1992). The differences in the ovarian responses to PMSG and pFSH could be attributed primarily to differences in the biological half-life of each gonadotrophin preparation (Armstrong *et al.*, 1983). PMSG has been used to superovulate cattle and sheep because of its availability, relatively low cost and ease of use, with only a single injection needed (Goulding *et al*., 1996). However, PMSG is known to have a relatively long circulating half-life as it remains active in the blood for 5 to 7 days after administration (Siddiqui *et al.*, 2002). The long circulating half-life of PMSG could result in the occurrence of excessive follicular development and failure of ovulation (Moor *et al*., 1985 and Murphy *et al*., 1991). Furthermore, given its capacity to bind to luteinising hormone (LH) receptors, PMSG exhibits LH-like activity in non-equine species and excessive LH activity in gonadotrophin treatments has been associated with follicular atresia (Tajima *et al*., 2007)) which is not an ideal effect for superovulation and embryo

recovery program. Even though PMSG stimulated ovaries prone to have failure in ovulation, it does not negatively affect the number of follicles recruited and oocyte retrieval via LOPU. The important concern of using PMSG which negatively affect the number of follicles and oocyte yield, however, is the provocation of anti-PMSG antibodies in repeated stimulation treatment. The presence of this anti-PMSG antibodies reduced caprine ovarian response towards PMSG resulting in lower follicular development and oocyte yield. Similar immunologically mediated ovarian refractoriness was also reported to occur in mice, rabbits, rhesus monkeys, cows and domestic cats that repeatedly injected with PMSG (Lin and Bailey, 1965; Onuma *et al*., 1969; Bavister *et al*., 1986; Swanson *et al*., 1996).

On the other hand, FSH either derived from sheep (oFSH) or swine (pFSH) has surpassed PMSG as the preferred gonadotrophin for goat superovulation because the stimulation and ovulation responses were reported higher in FSH- than in PMSG-treated goats (Armstrong *et al*., 1983; Tsunoda and Sugie, 1989; Goel and Agrawal, 1990; Pampoukidou *et al*., 1992; Pendelton *et al*., 1992). In contrast to PMSG, pFSH has a relatively short half-life and little LH-like activity. Thus, pFSH must be administered using multiple injection regime to ensure optimal superovulation. However, Riesenberg *et al*. (2001) reported that a single dose injection of FSH can be used to simplify the superovulation protocol, even though it is prone to premature luteal regression. In this study, in order to avoid the effect of single or multiple injection regime influencing the ovarian response, pFSH and PMSG was administered using multiple and single injection regimes, respectively.

In summary, results obtained from this experiment suggest that both PMSG and pFSH employed in the designated regimes have the potential to stimulate caprine ovaries for oocyte retrieval via LOPU. However, the efficacy of PMSG could not

surpass the pFSH, particularly, when it was employed in the repeated ovarian stimulation and oocyte retrieval programme.

5.2 EFFECT OF DIFFERENT SOURCES OF CAPRINE OOCYTES ON THE OOCYTE YIELD, GRADES AND MATURATION PERFORMANCE (EXPERIMENT 2)

This experiment was conducted to evaluate the effect of oocyte source (LOPU versus slicing of abattoir derived ovaries) on the oocyte yield, grades and maturation performance.

5.2.1 Effect of Two Different Sources of Caprine Oocytes on Quantity and Quality of Oocytes Obtained

In this present experiment, OR via LOPU was conducted after 71 hours of ovarian stimulation with pFSH coupled with hCG. The number of oocytes retrieved (11.0 per doe) via LOPU in this experiment was comparable to the results obtained by Abdullah *et al*. (2008), who obtained 11.4 and 16 oocytes per doe at 60 and 72 hours post-ovarian stimulation, respectively. The present finding is also in general agreement with the findings reported by Baldassarre and Karatzas (2004) and Gibbons *et al*. (2007) that the minimum number of oocytes yields via LOPU per doe - usually more than 5 oocytes.

In comparison to abattoir source, the number of oocytes retrieved via LOPU was significantly lower in this experiment. This is due to the fact that in LOPU, only follicles bigger than 2 to 3 mm were aspirated, but in abattoir ovary irrespective of size, all the follicles were sliced including those of smaller follicles of less than 2 mm in diameter. Martino *et al.* (1994) compared different oocyte collection techniques (dissection, aspiration and slicing) in order to obtain the largest number of oocytes for IVM-IVF programmes, they concluded that slicing is the most useful technique. However, the drawback of this technique is that it does not allow the selection of oocyte by follicular diameter that may be negatively affecting the subsequent *in vitro* meiotic competence of the oocyte retrieved as well as its embryo development.

Although OR by LOPU produced lower oocyte yield than slicing abattoir-derived ovaries source, this oocyte recovery procedure can be done repeatedly on the same donor without causing severe ovarian damage or decrease donor fertility. Moreover, unlike oocytes collected from abattoir-derived ovaries, LOPU-derived oocytes were retrieved from animals of known health status which could directly affect the oocyte yield in terms of the oocyte quantity and quality (Baldassarre *et al*., 2002). Due to the unavailability of records pertaining to breed, origin, health condition and phase of oestrus cycle for the does at the abattoir, there might be a possibility that the quality and quantity of the abattoir- derived oocytes obtained in this study affected by these factors.

The quality of oocytes yielded in this experiment was categorised according to the oocyte morphology and cumulus cell vestment (Rahman, 2008). The results obtained showed that, oocyte quality retrieved from LOPU was slightly better compared to those derived from abattoir ovaries. This was noted as the distribution of oocytes yielded via LOPU majority was in the categories of Grades A and B. Similar results

were obtained by Koeman *et al*. (2003) in which more than 80% of the caprine oocytes yielded via LOPU in their study were of Grades A and B. However, abattoir derived oocytes in this experiment, majority were distributed in the categories of Grades B and C. In other words, the preservation of cumulus vestments in the recovered oocytes via LOPU was good, suggesting that the needle size and the vacuum pressure applied during LOPU were properly selected (Baldassarre *et al*., 1994; 2003). In addition, the oocytes yielded via LOPU procedure were obtained from hormonally stimulated does in which the follicular growth was approaching preovulatory stage and logically, the COCs at this stage were less firmly attached to the follicle wall at the time of OR via LOPU. Thus, the cumulus cell vestment on the oocytes retrieved was not disrupted as lower vacuum pressure was required to detach the COCs from the follicle wall during LOPU. Besides that the ovarian stimulation treatment added credit to the quality of oocyte yielded via LOPU as they were homogenous during oocyte retrieval.

On the other hand, the quality of oocytes recovered from abattoir-derived ovaries was slightly compromised and heterogeneous, as the donors were not hormonally stimulated; thus, COCs collected were derived from follicles in different stages of growth and atresia (Rodríguez-González *et al*., 2002). Besides that several factors such as donor age, nutrition, season and genetic lineage are known to exert considerable influence on oocyte production and quality. Since the history and exact physiological state of oocyte donors from abattoir was unknown, the negative effect of the above mention factors might affect the quality of the oocytes yielded from abattoir-derived ovaries (Brackett *et al*., 1992; Martins *et al*., 2011).

5.2.2 Determination of an Optimised IVM Time Range for Caprine Oocytes Derived from Two Different Sources

The present experiment was undertaken to evaluate the time required by the LOPU- and abattoir-derived caprine oocytes to complete its nuclear maturation *in vitro* by observing the extrusion of the first polar body (PB-1). Besides that, localisation of the PB-1 and MII plate in the *in vitro* matured caprine oocytes were also investigated. At present, there were variations in the *in vitro* maturation (IVM) durations employed in several caprine IVP studies. Thus, there is yet a definite optimal IVM duration established for caprine oocyte maturation. In order to determine the optimum IVM duration for LOPU- and abattoir-derived oocytes in this experiment, a standard IVM medium formulation and selection of oocytes from only Grades A and B for both sources were used.

The 5 IVM durations (15, 18, 21, 24 and 27 hours) evaluated in this experiment were selected based on the range commonly used (16 to 27 hours) by other researchers (Cognié *et al*., 2003; Le Gal *et al*., 1992; Martino *et al*., 1994). The results obtained in this experiment showed that maturation of caprine oocytes retrieved from stimulated ovaries via LOPU was found to occur as early as 15 hours. However, the maturation rate at this hour was rather low (6.7%). Cognié *et al*. (2003) and Pawshe *et al*. (1994a) also reported that caprine oocytes derived from stimulated ovaries generally started to mature as early as 16 hours after the initiation of maturation process and completed at 24 hours. The IVM rate of caprine oocytes derived from stimulated ovaries in the present experiment peaked at 21 hours with the maturation rate of 86.7%. This IVM rate was comparable to the maturation rate obtained by Abdullah *et al*. (2008) (82.1%), and was relatively higher compared to those reported by Cognié *et al*. (2003) (75%) and Martino *et al*. (1995) (66.7%) who also used adult caprine oocytes derived from

stimulated ovaries in their studies. The relatively higher maturation rate reported in the present experiment and by Abdullah *et al*. (2008) might be attributed to the effect of prolonged interval from ovarian stimulation to LOPU (70 to 72 hours) as the interval used in the studies conducted by the other two research groups was shorter (48 hours).

Mammalian oocytes are known to undergoes significant changes to acquire meiotic competence while enclosed within the follicle and full meiotic competence could be achieved, especially when follicles becomes dominant and approaches ovulation (Hyttel *et al*., 1997). The effect of prolonged ovarian stimulation- LOPU intervals might enables oocytes to have sufficient time to acquired full meiotic competence, cytoplasmic as well as molecular maturation in the follicles. Once these oocytes are removed from its follicles, resumption of meiosis automatically commences and higher maturation potential might be attained by these oocytes (Sirard, 2001; Humbolt *et al*., 2005) compared to oocytes yielded from shorter ovarian stimulation-LOPU intervals.

Even though the effect of prolonged interval from ovarian stimulation to LOPU could be the reason towards the similarity of high maturation rate obtained in this experiment and by Abdullah *et al*. (2008), one prominent difference was observed between both studies is the IVM duration required to reach the optimal maturation rate. In this experiment, maturation rate of caprine LOPU oocyte peaked at a relatively shorter IVM duration (21 hours) compared to the IVM duration of 27 hours reported by Abdullah *et al*. (2008). The variation in the IVM duration reported might be affected by the differences in the IVM medium used in both studies. However, further studies are required to validate it.

It is notable that the ability to obtain high maturation rate in any embryo *in vitro* production (IVP) is one of the goals in IVP; however, the quality of the matured oocytes

obtained must not be ignored as this will affect significantly the subsequent embryo developmental competency. It has been suggested that spindle analysis could be used as the assessment parameter for the oocyte quality (Hu *et al.*, 2001, Eichenlaub-Ritter, 2002; Albertini, 2003). In this experiment, matured caprine oocytes were also obtained at 27 hours however, evaluation on the quality of the matured oocytes in terms of the morphology of PB1 and localisation of the PB-1 and MII spindle in these matured oocytes showed that more than 65% of these oocytes had polar bodies that either fully extruded (100% separated from the cytoplast) or with fragmentation and the MII spindle was observed to had translocated further apart from the PB-1. This may indicate negation in the oocyte development competence. Since evaluation on the morphology of PB-1 and localisation of the PB-1 and MII spindle was not carried out in previous study in our laboratory (Abdullah *et al.*, 2008), it is uncertain that the IVM duration of 27 hours reported was an ideal for their IVP. Hu *et al*. (2001) reported that prolongation of the IVM duration may further increase the yield of oocytes in MII as well as ageing of oocytes at MII in prolonged culture may cause concomitantly a deterioration of the spindle and displacement of the chromosomes.

Besides Abdullah *et al*. (2008), the studies conducted by Martino *et al*. (1995) and Gall *et al*. (1996) also incorporated 27 hours of IVM duration for goat oocytes derived from stimulated ovaries. Since the intervals from ovarian stimulation to LOPU in their studies were shorter (48 hours), it is rational that a longer IVM duration was required in their studies. However, further studies are needed to substantiate this phenomenon.

For abattoir-derived oocytes, maturation was observed to commence later at 21 hours and the maturation rate peaked at 24 hours with the rate of 80%. The abattoir-derived oocytes used slightly longer duration of IVM to reach MII phase compared to

LOPU-derived oocytes. This might be attributed to the fact that the abattoir–derived ovaries were not subjected to synchronisation and hormonal stimulation. Thus, the oocytes retrieved were heterogeneous, from follicles in different stages of growth and atresia (Rodríguez-González *et al*., 2002). When oocytes were retrieved from follicles less than 2 mm diameter and at pre-antral phase, the oocytes were yet to achieve full meiotic competency (Driancourt, 2001). Van den Hurk and Zhao (2005) also reported that the maturation rate of oocytes from late-stage follicles is more rapid than those from earlier-stages, however, oocyte yield from late-stage follicles are less abundant in non-stimulated ovary. Therefore it is reasonable that a slightly longer IVM duration might be required by the abattoir-derived oocytes to reach MII phase.

Even though, at the IVM duration of 27 hours, the maturation rate of abattoir-derived oocytes in this experiment (73.3%) slightly reduced, the rate obtained is still comparable to Martino *et al*. (1995) who obtained 75.9%. On the other hand, Sharma *et al*. (1996) reported that a rather longer IVM duration (32 hours) was required by the abattoir-derived oocytes to achieve the peak of the maturation rate (71.6%). Variations in the optimum IVM duration reported by Martino *et al*. (1995) and Sharma *et al*. (1996) compared to our study might be influenced by the differences in the culture medium components and conditions used for IVM.

Evaluation on the morphology of PB-1 and localisation of the PB-1 and MII spindle for *in vitro* matured LOPU-derived oocytes were made in this experiment. At the IVM durations of 18 and 21 hours, morphological evaluation on the PB-1 extrusion and its shape under the light microscope showed that more than 80% of the LOPU *in vitro* matured oocytes had PB-1 with smooth surface (normal matured oocyte) and was not fully extruded (80 to 90%) from the cytoplast. In addition, majority of the matured oocytes obtained at these IVM durations had PB-1 and MII spindle that were co-

localised. Similar morphology of PB-1 and localisation of the PB-1 and MII spindle were also portrayed by the abattoir-derived oocytes matured at the durations of 21 and 24 hours. On the other hand, both LOPU- and abattoir-derived oocytes matured at 27 hours showed that the MII spindle had translocated apart from the PB-1. Comparison on the observation obtained in this experiment with other researcher could not be carried out as to our understanding there were no study conducted up to date to evaluate morphology of PB-1 and localisation of the MII spindle in matured caprine oocytes.

It is worth to take note that the caprine *in vitro* matured oocytes obtained were subjected to nuclear transfer experiment, thus the location of MII spindle closed to PB-1 is important as it determines the success rate of enucleation using 'blind' squeezing technique as needed in this caprine intraspSCNT study. The 'blind' enucleation method was carried out by removing PB-1 and the underlying oocyte cytoplasm (10 to 15%), assuming that the MII spindle located adjacent to PB-1 from the matured oocyte in the absence of fluorochrome. Thus, it is important to carry out the enucleation step before the MII spindle translocated. Dominko *et al*. (2000) reported that displacement of MII spindle from its previously assumed location subjacent to the PB-1 in matured oocyte could affect the efficiency of enucleation, especially using 'blind' enucleation method. However, if fluorochrome labelled enucleation method was employed, displacement of MII spindle phenomenon in matured oocytes would not be an influencing factor towards the success of enucleation. Fluorochrome labelled enucleation can only be carried out provided that the fluorescent filter was attached to the micromanipulator. Since this setup was not available in our laboratory, fluorochrome labelled enucleation method could not be employed. In conjunction to this, evaluation on the appropriate maturation duration in which most of the matured oocytes did not show displacement of MII spindle from its PB-1 was conducted to maximise the success rate of the 'blind' enucleation in our experiment.

This research could be considered the first study conducted to examine the temporal and spatial relationships of the PB-1 and MII spindle on the matured caprine oocytes derived from two different sources (LOPU and abattoir). By taking into the consideration of the maturation rate, morphology and localisation of MII spindle obtained in this experiment, the findings of this experiment suggested that the optimal IVM duration range for LOPU- and abattoir-derived oocytes that could be employed in our laboratory are 18 to 22 hours and 22 to 26 hours, respectively.

5.2.3 Evaluation on the Meiotic Competency of Caprine Oocyte Derived from Two Different Sources According to Oocyte Quality

Evaluation on the meiotic competency according to oocyte quality for both LOPU- and abattoir-derived oocytes was conducted in this experiment. The LOPU- and abattoir-derived oocytes of different grades were subjected to IVM duration range of 18 to 22 hours and 22 to 26 hours, respectively, using the same formulation of IVM medium as depicted in Table 3.8. When the maturation rate was measured from the oocyte pool regardless of grades, the maturation rate of LOPU-derived oocytes was significantly ($P<0.05$) higher than abattoir-derived oocytes (79.6% versus 69.7%). This was in agreement with the study conducted by Rahman (2008).

Several investigations have revealed that the size of follicles and oocytes retrieved correlated positively with the oocytes meiotic competency (Richard and Sirard, 1996). In comparison to the LOPU-derived oocytes, abattoir-derived oocytes retrieved were heterogeneous in sizes. Due to the slicing technique of the abattoir- derived

ovaries, oocytes from smaller follicles (<2 mm) were also collected. Motlik *et al*. (1984) have demonstrated that oocytes retrieved from follicles of less than 2 mm diameter were less in meiotic competence. Thus, the lower maturation rate of the abattoir-derived oocytes compared to LOPU-derived oocytes might be attributed to the heterogeneity in oocyte sizes that were present from the abattoir source.

Both the maturation rates for LOPU- and abattoir-derived oocytes obtained in this experiment were slightly higher than that was obtained by Rahman *et al*. (2008) with the percentages of 73.8% and 54%, respectively. Variation in the maturation rates obtained in this experiment compared to Rahman *et al*. (2008) might be due to the differences in the IVM duration used and the chemical components supplemented into the IVM medium.

Even though the overall maturation rates of LOPU-derived oocytes were significantly ($P<0.05$) higher than abattoir-derived oocytes, when the comparison was made within each oocyte grades (Grades A, B, C and D), no significant difference ($P<0.05$) was observed in the maturation rate between LOPU- and abattoir-derived oocytes in their respective grades. The insignificance of the maturation rate observed within each oocyte grade was probably due to the relatively smaller sample size of the LOPU-derived oocytes compared to abattoir-derived oocytes subjected to IVM treatment in this experiment. The lower sample size of the LOPU source was due to the fact that the number of oocytes retrieved via LOPU per doe was usually lower compared to retrieval via slicing of abattoir-derived ovaries (Katska-Ksiaźkiewicz *et al*., 2004).

Oocyte quality is known to be an important prognostic factor to the nuclear and ooplasmic maturity of the oocyte which directly related to the success rate of embryo IVP (Kahraman *et al*., 2000). In this experiment, both LOPU- and abattoir-derived oocytes of Grades A (>5 layers of CCs investment), B (3-5 CCs layers) and C (1-2 CCs

layers) showed significantly ($P<0.05$) higher maturation competency compared to Grade D (<1 CCs layer). This showed that the presence of more than 1 layer of cumulus cells surrounding the COCs are essential in promoting the maturation of caprine oocytes regardless whether the COCs were retrieved from ovarian stimulated or non-stimulated ovaries. The presences of cumulus cells (CCs) are essential for oocyte growth, nuclear and cytoplasmic maturation as well as in genomic transcription activity (Van de Hurk and Zhao, 2005). No maturation or low rate of maturation in bovine (Fukui and Sakuma, 1980; Zhang *et al*., 1995) and sheep (Shirazi *et al*., 2007) oocytes was obtained when CCs were removed before the oocytes were matured *in vitro*. In addition, Goud *et al.* (1998) and Nyholt *et al*. (2009) also suggested that in human and monkey, successful IVM has only been achieved with cumulus-enclosed oocytes, indicating that it is crucial for IVM to remain COCs intact. The CCs investment plays a significant role in allowing nutrient transfer (Haghight and Van Winkle, 1990; Laurincik *et al*., 1992), energy substrates (Sutton *et al*., 2003) and/or messenger molecules for the development of oocyte (Buccione *et al*., 1990) and to mediate the effects of hormones on the COCs. Besides that, Bruynzeel *et al*. (1997) and Kastrop *et al.* (1991) also reported that CCs seems to be essential, especially in the first hours of maturation.

Based on the findings in this experiment, LOPU-derived oocytes approach seems to provide oocytes with better meiotic competency compared to the abattoir-derived oocytes. However, further study could be carried out to obtain a concrete conclusion by using a more homogeneous sample size for both the LOPU- and abattoir-derived oocytes. Besides that, a less varied source of abattoir-derived oocytes could be used by standardising the age, weight and health condition of the donor does in which the oocytes were to be collected.

5.3 PRODUCTION OF CLONED BOVINE AND GAUR EMBRYOS VIA INTRASPECIES AND INTERSPECIES SCNT APPROACHES: A PRELIMINARY STUDY FOR CAPRINE SCNT RESEARCH (EXPERIMENT 3)

The attempts to produce bovine-bovine intraspecies SCNT (intraspSCNT) and gaur-bovine interspecies SCNT (interspSCNT) were conducted as a preliminary experiment for the caprine SCNT research which was the core interest of this study. The manipulation efficiencies for bovine intraspSCNT and gaur interspSCNT in terms of the enucleation rate was reasonably high with the average of 90.3% compared to the report by Daniel *et al*. (2008) who obtained 60.7% of successful enucleation rate using the same blind squeezing method for caprine MII oocytes. A direct comparison on the success enucleation rate for bovine MII oocytes could not be done as majority of the research work reported on bovine NT does not indicate the enucleation rate in their study.

The fusion rates, cleavage rates and blastocyst rate of the bovine intraspSCNT embryos in this experiment (74.3%, 77.7%, 20.3%) were comparable to the study conducted by Cho *et al*. (2004) who obtained 79.9%, 67% and 15.2%, respectively using EF cells as donor karyoplast. However, when comparison was made with Srirattana *et al*. (2010) who obtained 91.7% of fusion rate, 90.1% of cleavage rate and 38.7% of blastocyst rate, the results obtained in this experiment was relatively lower, even though using the same type of donor karyoplast (EF cell), nuclear transfer protocol and IVC medium. The lower results obtained in this study compared to Srirattana *et al*. (2010) might be attributed to the relative disparities in operator skills and speed of manipulation (Yanagimachi, 2002; Perry and Wakayama, 2002) as the author of this

study were only exposed to the SCNT manipulation skill training for a short duration of 3 months.

As for gaur interspSCNT, the fusion rate and cleavage rate obtained in this experiment were comparable with the banteng interspSCNT experiment conducted by Sansinena *et al*. (2005) who obtained 77% and 67%, respectively. Due to limited information available on the fusion rate for gaur interspSCNT, a direct comparison on this data could not be made with other researcher doing on the similar species. Therefore, the fusion and cleavage rate of banteng-bovine interspSCNT which also having the similar taxonomic closeness as gaur-bovine interspSCNT was used as a comparison in this experiment. The percentage of gaur interspSCNT blastocyst obtained in this experiment (19.03%) was slightly higher compared to the percentage obtained by Lanza *et al*. (2000) and Mastromonaco *et al*. (2007) who obtained 12% and 11.8%, respectively. The differences in the percentage of blastocyst obtained might be due to the positive effect of the oviductal epithelial cells co-culture system used in the present experiment which was not employed in their studies.

When comparison was made between bovine intraspSCNT and gaur interspSCNT approach, there were no significant difference ($P>0.05$) in the fusion rate, cleavage rate and IVD rate (2-cell to hatched blastocyst) obtained in this experiment. This result trends were in agreement with the trend obtained by the senior researcher in ESRC, Suranaree University of Technology, Thailand in an unpublished report (K. Srirattana, personal communication). It is worth to note that the same optimal duration and field strength of electric pulsing (2 DC, 24 V, 15 µsec) were used for both bovine intraspSCNT and gaur interspSCNT in this experiment and interestingly the fusion rates obtained for both approaches were similar. This indicates that the gaur EF cell membrane properties are possibly compatible with bovine oocyte plasma membrane

(Dominko *et al*., 1999). In addition, the IVD competency of gaur interspSCNT embryos was comparable to the bovine intraspSCNT embryos in this experiment, and this reflects that bovine cytoplast is capable in reprogramming gaur EF cell. The report on the live birth of cloned gaur using interspSCNT approach by Lanza *et al*. (2000) proved that cloned gaur embryos generated using interspSCNT approach not only competent in the pre-implantation development but also competent at post-implantation after embryo transfer.

In general, the overall results in terms of the manipulation efficiency and IVD development of the reconstructed bovine intraspSCNT and gaur interspSCNT embryos obtained in this experiment using the SCNT protocol developed by ESRC Laboratory within just the short duration of skill acquisition were fairly convincing. This portrayed that the SCNT protocol developed by ESRC Laboratory for the production of cloned bovine and gaur embryos was effective, and could be used as a reference for the development of caprine SCNT protocol in the local laboratory setting (ABEL Laboratory). Due to species difference, modification of the adapted protocol was conducted in the following experimental design with the ultimate aim to develop a caprine SCNT protocol that could be used in ABEL Laboratory setting as well as in other laboratories in Malaysia.

5.4 IMPROVEMENT ON THE *IN VITRO* CLONED CAPRINE EMBRYOS PRODUCTION BY CONSIDERING THE EFFECTS OF MATURATION DURATION, ACTIVATION TREATMENT AND *IN VITRO* CULTURE PROTOCOL (EXPERIMENT 4)

5.4.1 Effect of Two Different IVM Intervals on Cloned Caprine IVD Competency using Ovarian-Superstimulated Caprine Oocyte

In this experiment, the manipulation efficiency and IVD competency of cloned caprine embryos generated using oocytes from stimulated ovaries matured at two different IVM intervals, namely 18 to 22 hours and 23 to 27 hours were evaluated. The oocytes from stimulated ovaries or known as LOPU-derived caprine oocytes, matured at 18 to 22 hours showed significantly ($P<0.05$) higher maturation rate, percentage of successful enucleation, cleavage and IVD rates compared to oocytes matured at 23 to 27 hours. The higher maturation and enucleation rate obtained for oocytes subjected to IVM duration of 18 to 22 hours further confirmed the findings in Experiment 4.2.2 in which, screening of optimum IVM duration for LOPU oocytes was conducted. The high enucleation rate correlated with the observation in Experiment 4.2.2 in which the location of MII spindle was closely adjacent with PB-1 in matured oocytes at the IVM duration of 18, 21 and before 24 hours. On the other hand, the lower maturation and enucleation rate for oocyte matured at 23 to 27 hours might be attributed to the prolonged MII arrest resulting in degenerated and fragmented PB-1 as well as translocation of the MII spindle apart from the PB-1 which affect the enumeration of matured oocyte based on the extrusion of PB-1 and the enucleation rate.

It is worthwhile to take note that in this experiment, exogenous LH was administered in the stimulation regime and a prolonged interval between hormonal (pFSH) stimulation and LOPU (70 to 72 hours) was adapted prior to OR. During the period between the LH surge and ovulation in the stimulated ovaries, oocyte is known to undergo a series of marked changes in its nucleus and cytoplasm towards maturation (Van den Hurk *et al*., 1999). Thus, there might be a possibility that the maturation process had begun to occur in the follicle prior OR in this experiment. After OR, it is logical that a relatively shorter IVM duration is required by the oocytes to reach MII phase as reflected in the high maturation rate obtained at 18 to 22 hours in this study. In comparison to the results of this experiment, the caprine intraspSCNT study conducted by Reggio *et al.* (2001) who used the same IVM duration of 18 to 22 hours, reported a relatively lower maturation rate and cleavage rate (50% and 57%, respectively). The low maturation and cleavage rate of oocytes matured at 18 to 22 hours could be attributed to the effect of the short interval between hormonal (pFSH) stimulation and LOPU (24 hours post pFSH administration) used in their study. The study conducted by Keefer *et al*. (2002) further support the findings that oocytes retrieved from short hormonal stimulation-LOPU interval required slightly longer IVM duration to yield a high maturation rate. In their caprine intraspSCNT study, oocytes retrieved from a short oFSH stimulation-LOPU (36 hours) interval protocol were subjected to a slightly longer IVM duration of 23 to 24 hours and the maturation rate obtained was high (94%). Even though the hormonal stimulation-LOPU interval and IVM duration seems to be a factor influencing the maturation rate, we could not rule out that the maturation rate also influenced by the formulation of IVM medium used. Thus, further refinement studies using a standard IVM medium are required in order to validate the interpretation of the effect of hormonal stimulation-LOPU interval and IVM duration on the maturation rate.

In regards to the IVD competency, the caprine reconstructed oocyte completing IVM early are more competent to develop to the morula stage than those completing IVM at a longer duration. This was indicated by the present results that significantly higher cleavage and morula rates were obtained from reconstructed oocytes matured at 18 to 22 hours compared to 23 to 27 hours. It is known that the duration of IVM could affect the competency of embryo development (Hölker *et al*., 2005). Inappropriate timing of maturation was reported to result in oocyte aging (Hunter, 1989; Hunter and Greve, 1997), abnormal chromatin decondensation (Dominko and First, 1997) and ultimately reduced the embryo developmental competency (Marston and Chang, 1964).

Oocyte age was found to influence the activity of maturation or M-phase promoting factor (MPF) and the IVF competency of oocytes (Kikuchi *et al*., 2000 and Grupen *et al*., 1997). During the process of oocyte maturation, the activity of MPF increased and remained at a high level during the meiotic arrest. However, when oocyte age prolonged, the MPF activity gradually decreased and the activation ability and fragmentation frequency of the oocyte cytoplasm was found to increase (Kikuchi *et al*., 1999, 2000). Thus oocytes matured at 23 to 27 hours in this experiment might be showing sign of oocyte aging gradually since the trend of IVD rate rapidly decreased from 2-cell to morula . Furthermore, the cleavage and IVD rate of these reconstructed oocytes were lower compared to oocyte matured at 18 to 22 hours at any cell stages. The interpretation of this observation was further strengthened with the findings by Yang *et al*. (1993) and Presicce and Yang (1994) that aged oocytes even though have high activation rates, the developmental potential of these oocytes were lower. In addition to this, Takano *et al*. (1993) and Tanaka and Kanagawa (1997) also reported that reconstructed oocytes cloned with young recipient oocytes were prone to produce embryos with a higher developmental rate.

When comparison was made with other caprine IVF and ICSI studies conducted in the present laboratory (Chan, 2008; Rahman, 2008; Kong, 2010) using the same IVC system, the IVP rate of caprine embryos obtained in this experiment using LOPU-derived oocytes matured early (18 to 22 hours) is higher compared to the results using oocytes matured later (27 hours) in their studies. Even though none of the IVP caprine embryos managed to develop beyond morula stage in this experiment as well as in the above mentioned IVF and ICSI studies, the relatively higher IVD rate up to morula stage obtained in this experiment suggested that the IVP of caprine embryos could be improved by using LOPU oocytes that matured earlier (18 to 22 hours). The author believes that the findings of this experiment has contributed one-step closer in solving the problem of caprine *in vitro* blastocyst production in the laboratory before further evaluating and refining the activation treatment and IVC system in the subsequent experimental designs.

5.4.2 Effect of Two Different Activation Protocols on the *In Vitro* Developmental Competency of Reconstructed Caprine Embryos

The present experiment was conducted to compare the effectiveness of two different sequential activation protocols on the IVD competency of reconstructed caprine oocytes following SCNT. The two sequential activation protocols investigated were (a) 7% ethanol (EtOH) + cytochalasin D plus cylocheximide (CD-CHX) and (b) calcium ionophore (CaI) + 6-dimethylaminopurine (6-DMAP). These two combinations of chemical activation agents were selected because they are commonly used, and are known to be effective in inducing bovine and caprine parthenogenetic as well as

reconstructed cloned oocytes (Shen *et al*., 2008; Laowtammathron *et al*., 2005; Ongeri *et al*., 2001; Apimeteetumrong *et al*., 2004). In additional, since the 7% EtOH + CD-CHX protocol was used in the preliminary experiment of this caprine SCNT research, and its efficiency was proven by the development of bovine blastocyst, thus it is worth to compare this activation protocol with the CaI + 6-DMAP protocol which is commonly used in caprine intraspSCNT research worldwide. To our understanding, this experiment is the first attempt in using 7% EtOH + CD-CHX to induce the development of caprine reconstructed oocytes.

The results obtained in this experiment demonstrated that both the 7% EtOH + CD-CHX and CaI + 6-DMAP activation protocols had the same efficiency in inducing the development of caprine reconstructed embryos since the IVD rates were similar at each embryonic stages. The high cleavage rate obtained in both activation protocols tested also indicated that the combinations of activation chemicals (7% EtOH + CD-CHX) and (CaI + 6-DMAP) in each protocol were favourable. Furthermore, when reconstructed caprine oocytes were activated with other combinations of chemical activation such as electrical pulse (EP) + 6-DMAP (Shen *et al*., 2006), EP + CB (Melican *et al*., 2005), EP + CD-CHX (Daniel *et al*., 2008) and Ionomycin + 6-DMAP (Reggio *et al*., 2001; Tang *et al*., 2011), the cleavage rates (25% to 65%) reported seems to be lower compared to the results obtained in this experiment (78% to 80%). The effect of both the activation protocols on the late stage of cloned caprine embryo *in vitro* development could not be compared with the above mention research groups as they conducted embryo transfer at 1 to 2-cell stage, except Tang *et al*. (2011) who cultured the reconstructed embryos up to day 7.

Even though in this experiment, the reconstructed oocytes activated using both activation protocols failed to develop beyond morula stage, the percentage of morula

obtained (42.8%) is relatively higher compared to the study conducted by Tang *et al*. (2011) who obtained 31%. Failure in developing reconstructed blastocyst in this experiment compared to Tang *et al*. (2011) and Liu *et al*. (2011) might be attributed to slight difference in activation chemical employed as they used ionomycin instead of CaI to generate initial calcium transient (Ware *et al*., 1989). Moreover, both the research groups used foetal fibroblast cell as donor karyoplast and the IVC medium as well as culture apparatus used in their study differ from the present experiment. Thus, the reason that the reconstructed oocyte failed to develop to blastocyst stage might not be solely due to the activation protocols examined in this experiment as the suboptimal IVC medium and system might be affecting the blastocyst development as well (Han *et al*., 2003).

It is known that an increase in intracellular Ca^{2+} concentration is a universal response elicited by the sperm during fertilisation of the oocyte (Whitacker and Patel, 1990). Given the importance of the Ca^{2+} release during fertilisation, most of the currently used oocyte activation procedures rely on methods to induce an intracellular Ca^{2+} increase. The capability of Ca^{2+} rises to release oocytes from the meiotic arrest is related to the ability to trigger persistent inactivation of maturation promoting factor (MPF) and cytostatic factor (CSF), which is the result of c-mos and mitogen activated protein kinase (MAPK) activity (Lorca *et al*., 1993; Collas *et al*., 1993). There are several chemical substances that could be used to induce Ca^{2+} oscillation even without penetration of sperm such as ionomycin (Loi *et al*., 1998), ethanol (Loi *et al*., 1998), calcium ionophore A23187 (Liu *et al*., 2002) and strontium (Cuthbertson *et al*., 1981).

It was demonstrated that any activation procedures that evoke only a single Ca^{2+} rise is not adequate for full oocyte activation as it causes only a transient decline of MPF and CSF activity (Lorca *et al*., 1993; Collas *et al*., 1993). Based on these,

alternative methods of activation have been developed that combine a transient inactivation of MPF obtainable with a single [Ca^{2+}]i rise, with a persistent inhibition of MPF, induced by addition of either protein synthesis inhibitors (e.g., CHX; Zakhartchenko *et al*., 1999) or non-specific kinase inhibitors (e.g., 6-DMAP; Liu *et al*., 1998). Protein synthesis inhibitors usually restrict the synthesis or re-accumulation of cyclin B, thereby, blocking the re-synthesis of MPF activity (Presicce and Yang, 1994). Non-specific protein kinase inhibitors inhibit kinase activity of MPF, by inactivating MAPK (Motlik *et al*., 1998; Gordo *et al*., 2000).

In the activation protocol using 7% EtOH + CD- CHX, the ethanol (EtOH) served to induce Ca^{2+} elevation that would inactivate the existing cytostatic factor (CSF), the subsequent cycloheximide exposure served to prevent renewal of CSF synthesis in the oocyte and CD in turns inhibited the microfilament polymerisation. While for the combination treatment of CaI + 6-DMAP, the CaI first initiated a calcium flux and then inhibited protein phosphorylation that induces pronuclei formation without completion of meiosis. Even though both the 7% EtOH + CD-CHX and CaI + 6-DMAP activation protocols activated the reconstructed oocytes in different ways, these two sequential activation protocols fulfilled the mechanism of inducing calcium oscillation and preventing calcium levels from dropping before MPF inactivation takes place which is important for activation to take place. Thus, both activation protocols in this experiment portrayed similar efficiency as evident by the similarity in cleavage and morula rate obtained in this experiment since the mechanism of activation was fulfilled. The efficiency of both the combination of activation protocols tested were further confirmed as, other research groups employing either 7% EtOH + CHX or CaI + 6-DMAP also reported to obtain high rate of pronulear formation, blastocyst and even offspring in their study (Presicce and Yang, 1994; Zakhartchenko *et al*., 1999; Loi *et al*., 1998; Liu *et al*., 1998; Cibelli *et al*., 1998). However, the chromosomal analysis of the

embryos generated by these two activation protocols are suggested for future study as there were controversial in reports that the use of activation substances could affect the post-implantation of embryos after embryo transfer (Bhak *et al*., 2006).

Since the reports on successful production of cloned kids and calf also employed CaI + 6-DMAP protocols (Keefer *et al*., 2001, 2002; Reggio *et al*., 2001; Lan *et al*., 2006; Shen *et al*., 2008) and due to the simplicity and shorter activation duration employed in the CaI + 6-DMAP protocols compared to 7% EtOH +CD-CHX, the former activation protocol was employed in the subsequent experiments to produce cloned and parthenogenesis caprine embryos.

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5.4.3 Effect of Two Different *In Vitro* Culture Media on the *In Vitro* Developmental Competency of Reconstructed Caprine Embryos

In this experiment, the effect of two different basic culture media on the IVD of caprine intraspSCNT embryos was compared. The basic culture media evaluated were modified synthetic oviduct fluid medium with amino acid (mSOFaa) and potassium simplex optimisation medium with amino acid (KSOMaa). mSOFaa had been used as a standard medium in in this laboratory (ABEL Laboratory) for IVP of caprine embryos (Phua, 2006; Chan, 2008; Rahman, 2008 and Kong, 2010) and it is also commonly used in other laboratory to culture IVF, ICSI as well as SCNT- derived caprine embryos (Keskintepe *et al*., 1998; Jiménez-Macedo *et al*., 2006; Romaguera *et al*., 2011; Tang *et al*., 2011). The outcome of the embryo development using this basal culture medium varies from one laboratory to another as modification by supplementing additional

substances as well as the differences in the incubation environment was implemented according to each laboratory setting.

The previous studies conducted in this laboratory, using the IVC system incorporating mSOFaa as basal culture medium could not support the IVD of caprine IVF and ICSI embryo up to blastocyst stage. Even in Experiment 4.4.1 and 4.4.2, none of the cloned caprine embryos manage to develop beyond morula stage using the similar culture medium. Thus, in this experiment, the efficiency of a new basal culture medium known as KSOMaa was evaluated as an attempt to improve the current IVC system in the laboratory for the production of *in vitro* caprine blastocyst. KSOM was originally formulated by Lawitts and Biggers (1991) for mouse embryo culture. An improvement on this medium, mKSOMaa, was formulated by Summers *et al*. (1995, 2000) with the addition of glucose (5.56 mM), BSA (4 mg/ml) and amino acids to increase the development rate of mouse IVF embryos. Since mKSOMaa supports mouse embryo development *in vitro* favorably. This culture medium was then tested in other domestic animals such as cattle (Bhuiyan *et al.*, 2004; Nedambale *et al*., 2004) and pig (Hashem *et al*., 2006) using a low glucose concentration (0.2 mM) and it seems that KSOMaa basal medium could support the *in vitro* development of cattle and pig embryos up to blastocyst stage. To our understanding, this experiment is the first attempt in using KSOMaa as the basal culture medium for caprine embryos IVC. Thus, in this study a comparison was made between the efficiency of using mSOFaa versus KSOMaa on the IVD of caprine intraspSCNT embryos.

The results obtained in this experiment demonstrated that KSOMaa basal medium has better efficiency compared to mSOFaa in supporting the IVD of cloned caprine embryos. This was evident as the cleavage rate, 8-cell and morula rate of cloned caprine embryos in KSOMaa were significantly ($P<0.05$) higher compared to mSOFaa.

Additionally, only embryos cultured in KSOMaa managed to develop to blastocyst stage.

It is worth to note that in this experiment, both mSOFaa and KSOMaa medium were supplemented with BSA-FAF and changing of culture medium during the embryo IVC was conducted at Days 2, 4, 5, 6, 7 post-activation. Thus, the efficiency of both mSOFaa and KSOMaa culture medium was not affected by the type of albumin supplemented but rather the chemical components used in the formulation of the basal media. When comparison was made in the chemical components between mSOFaa and KSOMaa, there were two distinct chemical components identified namely, EDTA and glucose that were only present in KSOMaa. These two distinct chemical components are likely to be the factors that differentiate the efficiency of both the basal media.

The IVD rates of caprine embryos at early cell stages cultured in KSOMaa in this experiment has significantly improved compared to the embryos cultured in mSOFaa. The improvement could be due to the presence of EDTA in KSOMaa. Similar observation on the beneficial effect of EDTA during early stages of embryos development was reported in murine (Gardner *et al*., 1996; Orsi and Leese, 2001) and bovine (Gardner *et al*., 2000; Olson and Seidel, 2000) embryos. It is well known that EDTA plays an important role in metabolic manipulation by depressing glycolysis during embryo pre-compaction, whereby high glycolytic rate is detrimental for embryo development (Thompson, 2000). Furthermore, EDTA also sequesters the toxic effects of heavy metal cations by inhibiting the production of reactive oxygen species, catalysed by ions such as Fe^{2+} and Cu^{2+} in the embryo IVC system (Johnson and Nasr-Esfahini, 1994).

Even though EDTA is known to be beneficial for embryo culture, prolonged exposure of embryos to EDTA in IVC medium is detrimental as reported by Brinster

(1965) and Gardner *et al.* (2000). They also found that *in vitro* development of post-compaction embryos depends on energy derived from high glycolytic activity; therefore, presence of EDTA in the medium during later stages of development will inhibit glycolysis, thus is harmful for the *in vitro* development of blastocyst. However when comparison was made on the concentration of EDTA used in the study describe by Gardner *et al*. (2000) and this present study, the concentration of EDTA (0.01 mM) used in this study was lower compared to that reported by Gardner *et al*. (2000) (0.1 mM). It is uncertain whether the lower concentration of EDTA in this study has positive effect on blastocyst production.

Besides that, the presence of glucose in KSOMaa might be the factor affecting the development of the cloned caprine embryos. The requirement for glucose in the culture medium for early stages of embryo development is controversial. Some reports stated that the presence of glucose during the first day of IVC is unfavourable to embryos in several species, namely mouse (Chatot *et al.*, 1989), sheep (Thompson *et al.*, 1992) and cattle (Ellington *et al.*, 1990). However, in this experiment high cleavage rate was obtained even though embryos were cultured in KSOMaa that contain glucose at the concentration of 0.2 mM. This result is in accordance with the findings by Matsuyama *et al*. (1993) who suggested that the presence of a low level of glucose 0.18 mM in the IVC medium for Day 0 to 3 is preferable for the *in vitro* development of bovine embryos. Perhaps, the concentration of glucose is the main factor that causes all the controversial among reports on the detrimental effect of glucose during the early stage of embryo culture. It is worth to note that, at Days 0 to 3 of the embryo *in vitro* development, even though glucose is not being used as the main energy substrate, it is known that it plays an important biosynthetic role in nucleic acid production (Rieger, 1992), which is essential for embryonic development at this early stage. Thus, the presence of trace amount of glucose in KSOMaa might be giving the beneficial effect to

the development of the cloned caprine embryos compared to the mSOFaa medium without glucose used in this experiment.

When comparison was made on the efficacy of mSOFaa used in this experiment and other research groups (Keskintepe *et al*., 1998; Jiménez-Macedo *et al*., 2006; Tang *et al*., 2011; Liu *et al*., 2011), the mSOFaa in this experiment could not support the *in vitro* development of cloned caprine embryos up to blastocyst stage which is in contradict to the results obtained by other research groups mention earlier. The discrepancy in the efficiency of mSOFaa might be attributed to the presence of sodium citrate (0.5 mM) in the formulation of mSOFaa used by the other research group which was not supplemented in this experiment. Sodium citrate is known to promote positive embryotrophic effect on embryo development as it stimulates fatty acid synthesis (Goodridge, 1973). Besides that, it also function as a chelator of metal ions (e.g., Ca^{2+}), a feature that may be of importance for maintaining junctional integrity and thus, of importance for compaction and blastocoel formation (Gary *et al*., 1992).

Even though, in this experiment, KSOMaa could support the development of cloned caprine embryos up to blastocyst stage, the blastocyst rate obtained was still low. Thus, modification on the formulation of KSOMaa was conducted in the subsequent experimental design as an effort to improve the blastocyst rate of cloned caprine embryos. The findings of this study has significantly contributed to the *in vitro* culture work of caprine embryos for this laboratory as this experiment was the first to successfully produced caprine blastocyst *in vitro* by using KSOMaa.

5.4.4 Effect of Increasing Glucose Concentration in KSOMaa medium at Day 2 culture on the *In Vitro* Developmental Competency of Reconstructed Caprine Embryos

As an attempt to further improve the *in vitro* cloned caprine blastocyst rate, a two-steps culture system was incorporated in this experiment by increasing the glucose concentration in KSOMaa medium at Day 2 culture (Treatment B). The efficacy of increasing glucose concentration in KSOMaa medium was evaluated by comparing the IVD and hatched blastocyst rate between embryos cultured in the one-step culture system of KSOMaa (Treatment A) and the two-step culture system (Treatment B) mentioned above.

In any culture medium, energy substrate is known to be one of the important ingredients for optimum *in vitro* development of embryos. Thus in this study, modification on the glucose concentration was conducted to optimise the *in vitro* development of cloned caprine embryos in particular blastocyst rate. At present, there are variations in the optimum concentration of glucose employed for different species and from one laboratory to another. Furnus *et al*. (1997) reported that supplementation of glucose concentration at 1.5 mM onwards in mSOF was effective in increasing bovine blastocyst rate, while raising the concentration to 5 mM is detrimental. This finding is in contrast to the report by Kim *et al*. (1993) who reported that glucose at 2.78 to 5.56 mM is favorable for increasing bovine blastocyst rates. In a study on mouse embryo culture, Summers *et al*. (1995) reported that glucose at 5.56 mM does not significantly inhibit blastocyst development. Thus, in view of widely varying reports on the optimal glucose concentration in different species, for the present study the author selected a glucose concentration of 2.78 mM supplemented in KSOMaa B which is

intermediate among the three reports mentioned above and compared it to the basal glucose concentration of 0.2 mM in KSOMaa A.

The results obtained in this study demonstrated that by increasing glucose concentration to 2.78 mM in Treatment B, the *in vitro* development of late embryo stages, (morula to blastocyst) significantly increased compared to embryos in Treatment A in which glucose concentration was not increased at Day 2 culture. The favorable effect of glucose increment in Day 2 culture was even prominent when the blastocysts cultured in Treatment B manage to hatch. The IVD rates of cloned caprine embryos at 2-cell to 8-cell stages cultured in both Treatment A and B did not differ significantly, as expected because for the first 2 days of IVC, Treatments A and B shared the same formulation of KSOMaa.

Increasing the glucose concentration from 0.2 mM to 2.78 mM on Day 2 for embryo culture favored development of cloned caprine embryos as reflected in the percentages of morulae and blastocysts obtained. Telford *et al*. (1990) and Rieger *et al*. (1992a), studying changes in the metabolism of glucose by bovine embryos throughout *in vitro* development, demonstrated that glucose metabolism does increase between the 8-cell and 16-cell stage, which is the time of embryonic genome activation. Similar observation on the marked glucose intake at the maternal zygotic transition phase of sheep (2 to 8- cell) and pig (4 to 8- cell) were reported (Thompson *et al*., 1991; Flood and Wiebold, 1988). Thus, the additional glucose incorporated into KSOMaa at Day 2 in this study may be rationalised as promoting better maternal-zygotic transition competence in cloned caprine embryos. Furthermore, glucose is also beneficial for blastocyst expansion because energy derived from glycolysis is required for maintaining the blastocoel (Rieger *et al*., 1992b; Benos and Balaban, 1990). This explains why, in Treatment A without additional glucose, the percentage of cloned caprine blastocysts

was significantly lower than in Treatment B and furthermore none of the blastocyst in Treatment A managed to hatch.

The cloned caprine blastocyst rate obtained in IVC Treatment B (19.9%) was comparable with other caprine intraspSCNT research groups such as Apimeteetumrong *et al*. (2004), Liu *et al*. (2007), Tang *et al*. (2011); Liu *et al*. (2011) who obtained average blastocyst range from 9.5% to 24% using different culture medium. Thus, this suggest that the two-step culture system (Treatment B) designed in this experiment is as effective as other culture system in use for caprine intraspSCNT research.

5.5 EFFICACY OF PRODUCING CLONED CAPRINE EMBRYOS USING INTRASPECIES VERSUS INTERSPECIES SCNT APPROACHES (EXPERIMENT 5)

This pioneering experiment was undertaken to produce caprine intraspecies SCNT (intraspSCNT) and interspecies SCNT (interspSCNT) embryos using KSOMaa as IVC medium in an effort to produce blastocyst. Besides that, it is believed that this experiment is the first attempt to produce cloned caprine interspSCNT embryo using caprine ear skin fibroblast cell as donor karyoplast and bovine oocyte as recipient cytoplast. The efficiency of caprine interspSCNT approach was compared with the intraspSCNT approach by evaluating the developmental ability, quality of blastocyst and the capability of post-implantation. The quality of blastocyst was evaluated in terms of hatching ability and total nuclei number. As a control, these parameters were also

evaluated for parthenogenetic activation (PA) embryos as Gupta *et al.* (2008) and Hong *et al*. (2005) had reported that the development characteristics of PA embryos resembled those of *in vitro* fertilised embryos and could be considered as a good model system to evaluate the influence of maternal factor (oocyte) on embryonic development by avoiding the confounding variation due to the influence of male/ sperm factor.

When the developmental competency of caprine intraspSCNT and caprine PA embryos were compared in this experiment, there were no significant difference ($P>0.05$) in the rate of cleavage and the development to the blastocyst stage (78% versus 74%, and 17% versus 15%). Both caprine intraspSCNT and PA blastocyst managed to proceed to hatched blastocyst stage with no significant difference in the hatching rate. Both IVD competency of caprine interspSCNT and bovine PA embryos showed similar trend in the cleavage rate (78% versus 73%), blastocyst rate (8.4% versus 12.6%) and hatched blastocyst rate (4.8% versus 7.9%) as obtained in the caprine intraspSCNT approach. The similarity in the IVD rate of intraspSCNT or interspSCNT versus the PA embryos indicated that the caprine ear skin fibroblast cell may be sufficiently reprogrammed in the recipient oocytes (Lu *et al.*, 2005), and it also indirectly showed that the manipulation technique employed during this SCNT study was reliable and did not impact negatively the *in vitro* development of the cloned caprine embryos.

The ability of caprine interspSCNT embryos developed to blastocyst stage (8.54%) in this experiment portrayed that skin fibroblast cell from caprine could be reprogrammed by enucleated bovine oocyte. Similar observation was also reported by Dominko *et al*. (1999), in which 13.9 to 16.6% of embryos reconstructed by transfer of the skin fibroblast cells from sheep, pigs, monkeys and rats into bovine cytoplasts developed to blastocysts. This showed that bovine cytoplasm could support the nuclei of

various species to the early cleavage stages and beyond, suggesting that it has the ability to reprogramme, at least partially, diverse donor nuclei past the maternal-embryonic transition (Dominko *et al*., 1999; Lanza *et al*., 2000; Kitiyanant *et al*., 2001; Sansinena *et al*., 2005).

When comparison was made with the caprine interspSCNT studies conducted by other research groups, the blastocyst rate obtained in this experiment (8.5%) was slightly higher than the caprine interspSCNT (2.2%) using buffalo cytoplast (Selokar *et al*., 2011) and was comparable to the caprine interspSCNT (7.4%) using ovine cytoplast (Ma *et al*., 2008). It is known that oocytes from different species may differ in their developmental competence, and this was shown in the study conducted by Lu *et al*. (2005) that buffalo oocytes were less efficacious than bovine oocytes in their ability to support the embryonic development of either buffalo or bovine fibroblasts after nuclear transfer (NT). Thus, the variations in the blastocyst rates observed in the comparative evaluation on the caprine interspSCNT mentioned above might indicate that bovine and ovine oocytes have a better efficacy than buffalo oocytes in supporting the embryonic development of caprine ear skin fibroblast cell. Besides that, when comparison was made with Song *et al*. (2008) who transferred foetal fibroblast cell into bovine ooplasm, the blastocyst rate obtained in their study (7.9%) was comparable to this experiment; suggesting that ear skin fibroblast cell might has similar reprogramming ability as foetal fibroblast cell. Similar observation was reported by Kato *et al*. (2000), Jang *et al*. (2004) and Srirattana *et al*. (2010) that there was only little if any difference between foetal and adult fibroblast following NT in terms of blastocyst development. However, further studies by standardising the SCNT procedure including laboratory personnel skill are required in order to validate the effect of oocyte cytoplast and type of donor cell factors on the efficiency of caprine interspSCNT discussed earlier.

During the caprine intraspSCNT versus interspSCNT experiment, both caprine and bovine oocytes showed similar maturation rate. This reflects that the *in vitro* maturation medium used in this experiment was versatile to support the maturation of oocyte from two different species (caprine and bovine) used in this study. The fusion rate of interspSCNT approach was significantly lower than the intraspSCNT approach. Thus, this indicates that the fusion parameters applied in the caprine intraspSCNT experiment are not sufficient for the fusion of bovine cytoplast and modification in the fusion parameters for (caprine karyoplast-bovine cytoplast) interspSCNT is suggested for future study.

When the efficiency of intraspSCNT and interspSCNT approaches was compared, it seems that the bovine ooplasm has similar competency as caprine ooplasm in promoting dedifferentiation of caprine nuclei as well as in supporting the embryonic development prior maternal-zygotic transition (8-cell to morula). This was reflected by the non-significance difference in the cleavage rate and the developmental rate at the 8-cell stage between both approaches. However, when approaching the late embryo developmental stages (morula to blastocyst), the interspSCNT approach was not as efficient as intraspSCNT approach when significantly lower morula and blastocyst yield were obtained in caprine interspSCNT approach. Li *et al*. (2006a) suggested that a closer genetic background between the donor cell and recipient oocyte could enhance blastocyst development *in vitro*. This suggests that the low blastocyst rate in caprine interspSCNT might be due to the genetic distance between bovine and caprine, which might result in incompatible genomic regulation and metabolic mechanism. The birth of a live cloned gaur by interspSCNT (Lanza *et al*., 2000) and successful implantation of yak interspSCNT embryo (Li *et al*., 2007) using bovine oocytes as recipient cytoplasm suggested that a close phylogenetic distance between donor nucleus and recipient cytoplasm resulted in a higher likelihood of success in delivering live offspring. Even

though gaur offspring was produced following interspSCNT using bovine cytoplasm, declination in blastocyst yield was observed, suggesting that sufficient differences that exist between the bovine and gaur at genetic and molecular level could be accountable for differences in embryo growth dynamics (Mastromonaco *et al*., 2006).

It is worth to mention that, the ability of cloned caprine embryos derived from both intraspSCNT and interspSCNT approaches in this experiment managed to develop beyond morula stage was influenced by the culture system employed. This was evident as none of the caprine intraspSCNT and interspSCNT embryos managed to develop beyond morula stage in our previous study (Abdullah *et al*., 2011) until changing of a new culture system was employed by using the optimised two-step culture system (Treatment B) using KSOMaa as basal medium in this experiment. Even though the culture system developed in this study could support the development of caprine interspSCNT blastocyst, the probability and fate of these embryos to develop to term is still unknown.

In terms of the blastocyst quality evaluation, the cell number of caprine interspSCNT blastocysts seems to be lower than in caprine intraspSCNT and PA blastocysts. Similar observations were reported in other interspSCNT studies such as in buffalo-bovine cytoplast (Atabay *et al*., 2004), monkey-bovine cytoplast (Lorthongpanich *et al*., 2008) and bovine-buffalo cytoplast (Lu *et al*., 2005) interspSCNT. It is important to examine the blastocyst cell number because the developmental competence of embryos to term after embryo transfer correlates with the number of cells present in the blastocyst. Fleming *et al*. (2004) demonstrated that low blastocyst cell numbers are implicated in reduced development and may lead to large offspring syndrome.

At present, the available reports on the successful production of cloned caprine are through intraspSCNT (Baguisi *et al*., 1999; Keefer *et al*., 2002; Reggio *et al*., 2001), however, the overall pregnancy rate after transfer is still low. None of the domestic animals was successfully cloned using interspSCNT in particular intergeneric approach. Most of the cattle (Lu *et al*., 2005), buffalo (Kittiyanant *et al*., 2001), sheep (Dominko *et al*., 1999), pig (Uhm *et al*., 2007) and goat (Selokar *et al*., 2011) intergeneric SCNT embryos only managed to develop up to blastocyst stage. Similarly, in this experiment, no pregnancy was detected after 8 attempts of embryos transfer conducted on both caprine intraspSCNT and interspSCNT embryos. Failure in obtaining viable cloned kids might be attributed to fact that the number of intraspSCNT and interspSCNT embryos (4 to 8 embryos) transferred per recipient is not sufficient. Chen *et al*. (2007) and Liu *et al*. (2011) demonstrated that the pregnancy rates and kids born per recipient increased as more cloned caprine embryos (10 to 40 embryos) were transferred per female. This might be due to the fact that implantation competency of cloned embryos are rather low due to defects in the cloned placenta (Liu *et al*., 2011), thus by increasing the number of cloned embryos per transfer or co-transferred with parthenogenetic embryos, the probability of gaining pregnancy might increase. However, further investigation on this are required as there were also reports that viable cloned offspring was obtained by just transferring 1 to 4 cloned embryos into one surrogate mother (Baguisi *et al*., 1999; Tang *et al*., 2011). Besides that, the negative implantation rate of caprine interspSCNT embryos might also be due to the incompatibility of mitochondria and genomic DNA of donor nucleus (karyoplast) and recipient oocyte (cytoplast) which results in post-implantation defect. Since this experiment was the first attempt to transfer cloned caprine interspSCNT embryo into the surrogate mother, it was unsure whether using goat or bovine as surrogate mother would be more suitable for the interspSCNT embryos to develop to term. However, further studies on the factors described above as

well as increasing the number of embryo transfer attempts are required before a firm conclusion could be made on the post-implantation competency of the intraspSCNT and interspSCNT embryos produced in this research.

In a nutshell, both intraspSCNT and interspSCNT approaches could produce cloned caprine embryos to blastocyst stage using the protocol developed in this research. Even though both approaches could produce cloned caprine embryos, the efficiency of interspSCNT approach is still low compared to intraspSCNT approach.

5.6 GENERAL DISCUSSION

Generally, the overall production of cloned animals by SCNT involves multiple steps including oocyte collection, donor cell culture, oocyte maturation, enucleation, donor karyoplast transfer, activation, IVC and embryo transfer (Figure 5.1). As mentioned in the figure, both technical and biological factors underlying in each of these steps are known to influence the successful outcome of the overall SCNT performance. In this research, a comprehensive study to optimise all of the influencing factors in each steps of the SCNT depicted in Figure 5.1 could not be conducted due to the short time frame and furthermore, the present study was the first attempt of SCNT in this laboratory. Therefore, the author only selected a few factors targeting to optimise oocyte yield, oocyte maturation performance, activation treatments and IVC system in the present caprine intraspSCNT study with the vision that part of these findings could benefit other caprine embryo IVP research in the future.

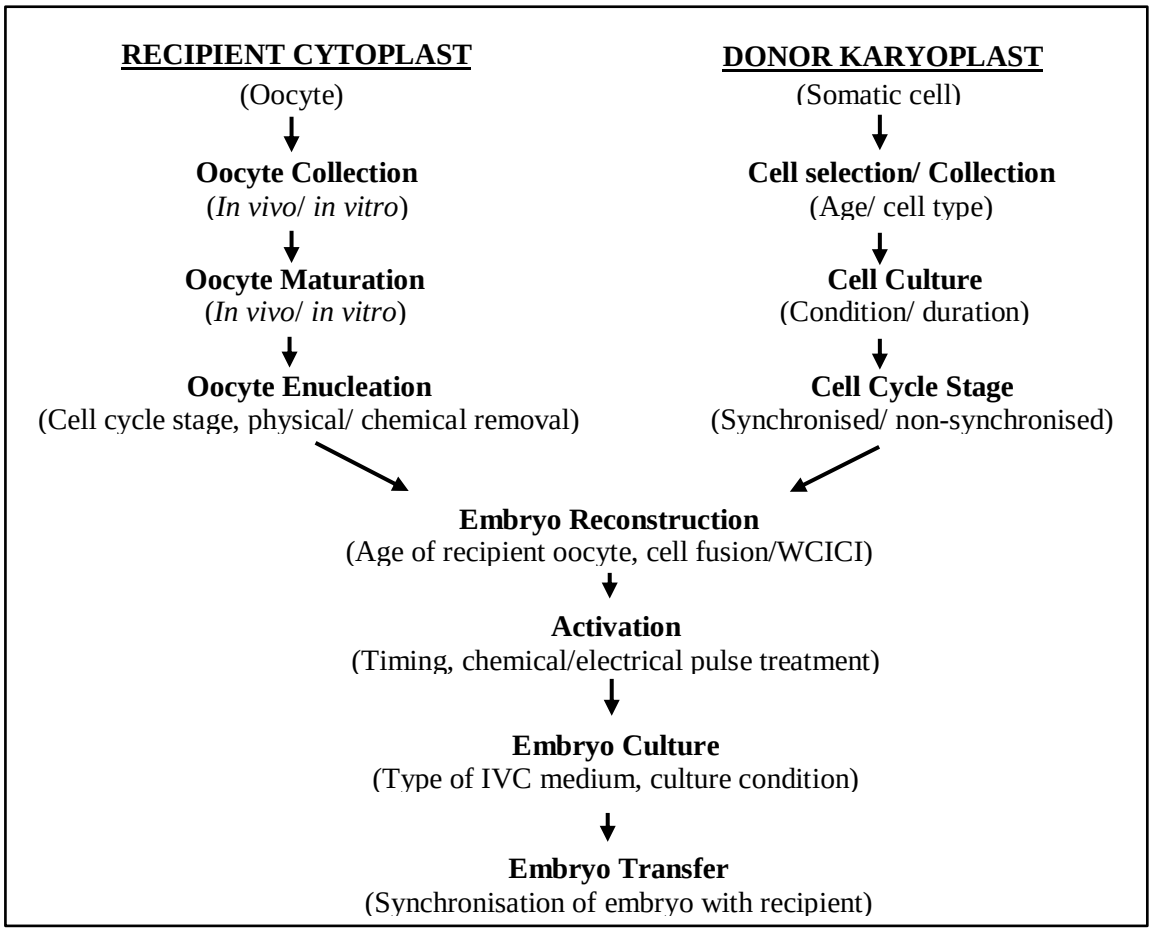

Figure 5.1: Flow chart of steps involved in SCNT and factors affecting each step.

Oocyte is known as the "Holy grail" in the embryo IVP study. With the current advances in the ART, embryo can still be produced *in vitro* using somatic cell via nuclear transfer approach, that is without involvement of conventional sperm fertilisation, Thus, availability of a constant and sufficient supply of oocytes is the prime factor that determines the efficiency of embryo production *in vitro*. In goat intraspSCNT, immature oocyte generally can be retrieved from live does via LOPU and abattoir-derived ovaries.

Oocytes from abattoir-derived ovaries have been the principle choice for the caprine IVP research due to the fact that it is inexpensive and readily available in almost unlimited quantities in some of the countries (Martino *et al*., 1994; Pawshe *et al*., 1994b; Ongeri *et al*., 2001; Rho *et al*., 2001). However, in Malaysia the source of caprine

oocytes from abattoir-derived ovaries is rather limited due to low slaughtering as a result of acute shortage of does in Malaysia (Rajikin, 1996). Moreover, slaughtering of does were often conducted in batches at only a selected period of time in a year, thus continuous weekly supply of goat ovaries from abattoir was not possible. To overcome this hurdle, LOPU is indeed an important alternative to obtain the supply of oocyte from live does in this local setting (Phua, 2006; Rahman, 2008; Kong, 2010). LOPU was proved to be an efficient and relatively non-invasive technique for OR from small ruminant like ewes (Baldassarre *et al*., 1994; Gibbons *et al*., 2007) and does (Baldassarre, 2002; Baldassarre and Karatzas, 2004; Gibbons *et al*., 2007) compared to transvaginal ultrasound-guided aspiration (TUGA) or laparotomy method. This is evident that LOPU may be repeated several times without causing ovarian damage or decrease in the donors' fertility (Stangl *et al*., 1999; Alberio *et al*., 2002; Pierson *et al*., 2005). Furthermore, LOPU after multi-dose hormonal treatment may be repeated (Tervit *et al*., 1992, 1993; Alberio *et al*., 2002), without diminishing good quality oocytes harvested (Tervit *et al*., 1992; Stangl *et al*., 1999; Alberio *et al*., 2002), which was also observed in this study after three repeated OR cycles.

Oocyte retrieval from live does using LOPU technique, often involves the application of oestrus synchronisation and ovarian superstimulation treatment on the live does to increase the number of follicle growth at a fixed time for OR. FSH from ovine (oFSH), porcine (pFSH) and PMSG or eCG are the commonly used gonadotrophins for livestock ovarian stimulation (Smartzi *et al*., 1995; Nowshari *et al*., 1992). A number of experiments have been performed to compare the superovulatory response between FSH and PMSG in livestock animals, the evidence favours the use of FSH than PMSG (Tsunada and sugie, 1989; Pendelton *et al*., 1992; Mahmood *et al*., 1991; Nowshari *et al*., 1992).

Does that were treated with PMSG was frequently associated with a high number of non-ovulated follicles, early regression of CL, short or irregular oestrous cycles and potential risk of embryo expulsion in MOET programme (Amoah and Gelaye, 1990). Furthermore, Tsunada and Sugie (1989) reported that the average number of oocytes recovered was significantly higher in FSH-treated does (9.4) than that in PMSG-treated ones (5.7). In contrast to the above findings, the present study showed that there were no significant difference in the average number of oocytes recovered via LOPU between FSH-treated (12) and PMSG-treated (11) does. Variation in the results obtained between the present study and Tsunoda and Sugie (1989) might be due to the differences in breeds, dosage and hormonal regime factors.

Even though PMSG was reported to negatively influence the superovulation treatment of livestock for embryo collection, especially the incident in which follicles fail to ovulate, this effect might not be a negative impact to the OR via LOPU programme as oocytes were retrieved from follicles prior ovulation. Furthermore, due to its lower cost, easy availability and it can be more easily administered (single injection) than FSH (multiple injection), perhaps PMSG can be a good choice to be used in OR via LOPU programme, if refinement on the regime targeting to minimise the effect of provocation of anti-PMSG antibodies in the repeated stimulation treatment is carried out. The decline in the ovarian response in the repeated stimulation treatment using PMSG was often associated with the existence of antibodies anti-PMSG accumulated in the animals. A decrease in the number of oocyte retrieved from PMSG-treated does at OR 3 was observed in this study, and this might be due to the provocation of anti-PMSG antibodies in the repeated stimulation treatment. However, Gibbon *et al.* (2007), in their study, found that neither follicle number nor oocyte recovery rate and viability were affected by the reiteration of the PMSG treatments in goats. A possible explanation to the variation in the observation might be related to the short time elapsed (4 days

interval) between treatments of LOPU on the stimulated does employed in their study as this time elapse might be too short for antibodies production. Perhaps in future, refinement of the present PMSG regime targeting on the time elapse between LOPUs and the incorporation of PMSG antiserum after PMSG injection could be carried out to improve the stimulatory response of PMSG used in the repeated LOPU sessions.

Oocyte retrieval from abattoir-derived ovaries produced a relatively higher number of oocyte yields than LOPU from stimulated does in this study. However, the quality and maturation rate of the oocytes retrieved from abattoir-derived ovaries were relatively lower than LOPU oocytes as observed in this study. According to Rahman *et al*. (2009), during IVM higher numbers of abattoir-derived oocytes died than LOPU source, which definitely affect the maturation rate of the abattoir-derived oocytes. Additionally, abattoir-derived oocytes were highly heterogeneous in nature and this heterogeneity came from different grades of growth and atresia of the oocytes obtained, which might have contributed to the lower maturity in some of these oocytes. On the other hand, in LOPU, as does were stimulated with hormones, and only follicles >2-3 mm were punctured, chances of survivability and maturity were better than with abattoir oocytes. In the present study, all the oocytes of heterogeneous grades were cultured including COCs with <1 complete CC layer due to a shortage of oocytes in both sources. However, other authors whom reported to produce high IVM rate (>70% to >80%) cultured good grades of oocytes with at least 4 (Jiménez-Macedo *et al*., 2006), 2 and above (Rho *et al*., 2001; Tajik and Esfandabadi, 2003), or 1-2 (Martino *et al*., 1995) layers of CC. Thus, the lower maturation rate reported in this study might be due to the quality of COCs cultured in particular the number of cumulus cell layer. It has been suggested that both the presence of cumulus cells and cell-oocyte contact are crucial for oocyte maturation to attain developmental competence (Leibfried-Rutledge *et al*., 1989; Gall *et al*., 2005). In addition, the developmental competence of bovine oocytes

surrounded with corona cells was induced in a cell density-dependent manner in the maturation medium (Hashimoto *et al*., 1998). This supporting mechanism has been shown to be partially due to the soluble factors secreted from cumulus cells (Hashimoto *et al*., 1998). Thus, in order to promote the maturation rate of oocytes with less than 1 complete layer of cumulus cells which are usually obtained from the pool of abattoir-derived oocytes, perhaps these oocytes can be cultured along with oocytes that consist of more than 2 layers of cumulus cell in the same droplet of IVM medium. Furthermore, additional of epidermal growth factor was also reported to enhance the maturation and developmental rates of abattoir- derived oocytes (Lonergan *et al*., 1996).

The *in vitro* developmental competency of cloned embryos could be influenced by the oocyte age (duration of MII arrest after IVM), activation treatment and the IVC system. Inappropriate timing of maturation could lead to oocyte aging (Hunter, 1989; Hunter and Greve, 1997), the formation of abnormal chromatin (Dominko and First, 1997) and impairment in embryo development (Marston and Chang, 1964). As the age of the oocytes at the time of MII arrest increased (extrusion of the polar body by 20 or 24 hours), a decrease in the ability of bovine oocytes to cleave and develop to the blastocyst stage was observed by Dominko and First (1997). In SCNT, the activation response in oocytes by several activation agents has been demonstrated to be oocyte age dependent.

Before the discovery of activation treatment using combination of chemicals and or electrical stimulation, the single activation treatment responded favourably on aged oocytes. Thus, in the 1990s, aged oocytes were preferably used in nuclear transfer procedure (Bondioli *et al*., 1990; Nagai *et al*., 1992; Barnes *et al*., 1993) as they were reported to respond well to the single activation treatment compared to young (freshly matured) oocyte (Yang *et al*., 1993; Presicce and Yang, 1994). When a combination

treatment of activation was used in young matured oocyte, the developmental ability of the embryos was reported to be even higher than aged oocyte activated using single activation treatment (Takano *et al*., 1993; Tanaka and Kanagawa, 1997). Ever since then, using of combined activation treatment is ideally incorporated in SCNT procedure, however, in order to obtain high IVD competency post-SCNT, young matured oocyte are required. In order to avoid oocyte aging factor influencing the IVD competency of cloned embryos, determination of an optimum IVM duration range prior SCNT was conducted in this study.

The finding in this study suggested that by using the IVM medium and incubation system in the present protocol, the optimum IVM duration range for LOPU-derived oocyte slightly shorter compared to abattoir-derived oocyte (18 to 22 hours versus 22 to 26 hours) in which at these IVM duration range, the maturation rate and percentage of successful enucleation was high as the location of MII spindle is closely adjacent to PB-1. Additionally, when LOPU-derived oocytes matured at a shorter IVM duration (18 to 22 hours) were used, the IVD rate of the reconstructed caprine embryos up to morula stage was significantly higher than the prolonged culture duration (23 to 27 hours). In fact, the percentage of morula obtained in this caprine intraspSCNT study using LOPU oocyte matured at 18 to 22 hours was relatively higher compared to the caprine IVF and ICSI study using LOPU oocyte matured at 27 hours in this laboratory (Chan, 2008; Rahman, 2008). The author speculated that the oocyte aging might be the factor influencing the embryo developmental rate as the same IVC system was used in all the studies compared above.

Activation procedures used in somatic cell nuclear transfer (SCNT) are one of the critical factors affecting the efficiency of animal cloning. Currently, combined treatment of two activation stimuli are commonly used in cloning studies to ensure

better development of reconstructed oocytes (cattle: Cibelli *et al*., 1998; goat: Keefer *et al*., 2001). The combined activation treatments are usually designed to reduce MPF activity in reconstructed oocytes, which is followed by treatment with a protein synthesis inhibitor combined with a broad spectrum protein kinase inhibitor to block the activity of newly synthesised MPF (Liu *et al*., 1998; Tian *et al*., 2002). The author found that, the activation response using combined chemical activation treatment of 7% EtOH + CD-CHX was comparable to the treatment of CaI + 6-DMAP in the caprine intraspSCNT study as the IVD rate of reconstructed embryos up to morula stage was similar in both treatments. In bovine intraspSCNT study, Bhak *et al*. (2006) reported that 6-DMAP treatment could result in an increased incidence of chromosomal abnormalities compared to the use of CHX. Even though 6-DMAP was reported to increase the incidence of chromosomal abnormalities, the bovine intraspSCNT embryos using either 6-DMAP or CHX produced similar blastocyst developmental rate and the total cell number was similar as well. Even though the activation response was similar for both chemical activation treatments in this study, the author had chosen the CaI + 6-DMAP activation protocol to be used in the overall caprine intraspSCNT and interspSCNT studies due to the fact that many reports on live cloned kids production used the similar activation treatment (Keefer *et al*., 2002; Tang *et al*., 2011) and in comparison to the 7% EtOH + CD-CHX treatment, the chosen treatment seems to be relatively convenient in the activation time and preparation. However, the cloned caprine embryos reconstructed using oocyte matured in the optimised IVM duration and activated with both treatments failed to developed to blastocyst stage, and similar *in vitro* developmental pattern was observed in the IVF and ICSI study conducted in this laboratory (Chan, 2008; Rahman, 2008; Kong, 2010).

Failure in obtaining caprine blastocyst development after SCNT and even in other previous IVF and ICSI studies conducted by other researchers in this laboratory

using the mSOFaa IVC medium for the past 7 years had been a big challenge and also the guiding light that indicated the need to improve or change the existing IVC system in this laboratory. There are various stressors in the IVC system that is reported to influence the *in vi*tro development of embryos such as inappropriate medium formulation, medium supplementation, the oxygen tension in the incubation system, and technical issues (Gardner, 2004). The utilisation of co-culture system and under low O_2 environment which mimic the physiological condition of the reproductive tract was reported to be beneficial for embryo development (Bavister, 1995; Edwards *et al*., 1997; Orsi and Reischl, 2007). Combination of both co-culture and low O_2 environment in the *in vitro* culture of embryo was employed in several embryo IVP studies which resulted in favourable blastocyst production rate (Nagao *et al*., 1994; Rodríguez-Dorta *et al*., 2007; Goovaerts *et al*., 2009; Srirattana *et al*., 2010). Incorporation of co-culture and low O_2 environment in the preliminary experiment of this research conducted in ESRC Laboratory, Thailand, resulted in the development of cloned bovine and gaur blastocyst as well. However, the drawback of using co-culture is it bears a considerable risk of contamination (Bavister, 1995). Even though in the preliminary experiment, this culture system was shown to support the development of cloned blastocyst, the deficiency in laboratory facilities and the difficulties in obtaining continuous supply of oviduct cells from cattle at the ovulatory phase in Malaysia, render the feasibility of using this IVC system in the caprine intraspSCNT and interspSCNT studies.

In order to improve the IVC system besides focusing on the co-culture and low O_2 culture system, the author substituted the use of mSOFaa basal medium with KSOMaa out of curiosity to test the IVD response of caprine embryos in this basal medium, since this medium was not used in any caprine IVC study. Interestingly, after incorporating KSOMaa in the IVC system, the reconstructed caprine embryos managed to develop up to blastocyst stage. However, the blastocyst rate obtained was lower

compared to other caprine intraspSCNT studies using mSOFaa as basal medium (Liu *et al*., 2011; Tang *et al*., 2011). For further improvement in the cloned caprine blastocyst rate, a two-step culture system was designated by increasing the glucose concentration in the KSOMaa for the IVC of late stage embryo development (second phase of IVC). The idea of increasing glucose supplementation in the two-step culture system designated in this study was based on the finding by Rieger *et al*. (1992) that the metabolism of glucose through the pentose-phosphate pathway increased almost 15 times and the total metabolism of glucose 30 times, during development of embryo from the two-cell to the expanded blastocyst stage. The first marked increase in glucose metabolism was reported to occur only at the embryo stage between the 8- and 16-cell stages in which the time of activation of the embryonic genome take place. In accordance to this, perhaps the increase in glucose concentration done at Day 2 post-activation in the designated IVC system supported the cloned caprine embryo development up to hatched blastocyst. The main achievement from this study was the finding of this new designated two-step culture system using KSOMaa as basal medium that could support the development of cloned caprine embryos up to blastocyst stage. Development of this IVC system is indeed a significant breakthrough for the production of caprine blastocyst *in vitro* regardless of using IVF, ICSI or SCNT approach in this present laboratory.

Most of the successful production of cloned animal often used the intraspSCNT approach (Wilmut *et al*., 1997; Kato *et al*., 1998; Baguisi *et al*., 1999). Even though the approach of interspSCNT are able to surmount the issue of acute oocyte supply of certain animal, particularly in saving the endangered animals, the overall efficiency of this approach is still low (Loi *et al*., 2011). It is important to note that in all the live offspring produced to date by interspSCNT, all the recipient oocytes were recovered from a species closely related to the one providing the donor somatic nuclei. It is

possible that incompatibilities between nuclear and ooplasmic components from more distant species are responsible for the limited success of the interspSCNT approach as observed in this study as well, in which the overall developmental competency of caprine interspSCNT embryos up to blastocyst stage was lower compared to intraspSCNT approach. Even though, the developmental rate of interspSCNT (caprine karyoplast-bovine cytoplast) embryos obtained in this study was low, it is worth mentioning that this study is first report in producing cloned caprine blastocyst using interspSCNT (caprine karyoplast-bovine cytoplast) approach.

5.6.1 Summary of Significant Findings

There were three novel findings achieved in this research which includes: (a) The optimal IVM duration range for LOPU-derived oocytes is slightly shorter (18 to 22 hours) compared to abattoir-derived oocytes (22 to 26 hours); (b) KSOMaa basal medium could support the development of caprine intraspSCNT and interspSCNT embryos to blastocyst stage. The additional supplementation of glucose into KSOMaa medium on Day 2 of IVC enhanced the hatching of blastocyst; (c) Cloned caprine blastocyst could be produced using interspSCNT (caprine karyoplast-bovine cytoplast) approach.

5.6.2 Constraints of the Studies and Future Directions

The present research was a preliminary study to develop a protocol for the production of cloned caprine blastocysts through intraspSCNT and interspSCNT approaches. During the development of this caprine SCNT protocol, emphasis was given more on the improvement of oocyte factors on the production aspect of cloned caprine embryos. Therefore, it was beyond the scope to study other aspects pertaining to donor cell, the

mechanism or process at molecular level, particularly on nuclear reprogramming in a great detail. Since this study was the first caprine SCNT research conducted in this laboratory as well as in Malaysia, many fundamental issues on caprine SCNT at the local setting had to be faced and surmounted.

Along this research journey, the difficulties encountered included factors such as:

a) Skill acquisition:

Intensive learning curve phase in microtools preparation and handling of the micromanipulator were endured at the early stages of the research (approximately 9 months) due to lack of facilities, skills and expertise in this laboratory in the area of cloning.

b) Oocyte supply:

Irregularity in obtaining abattoir-derived ovaries disrupted the completion of the sub-experiment according to the milestone set. To minimise this hurdle, OR from synchronised and stimulated does via LOPU was conducted, however, the consistency in the number of oocyte yield per replicate varied as factors such as age, breed, health and physiological status of oocyte donors could not be standardised and optimised throughout the research due to the shortage in the number of does available for selection and the sub-standard in the farm management level, especially in animal feeding.

c) IVM duration:

In the early attempt of intraspSCNT in this study, the IVM duration used for LOPU-derived oocyte was 27 hours. This duration was selected based on the duration for abattoir-derived oocytes which was generally used in other caprine

oocyte maturation study. Poor developmental competency was observed after using LOPU-derived oocytes matured at 27 hours in the early stage of this study. The author speculated that 27 hours might be slightly long for the MII arrest of the LOPU-derived oocyte which would cause oocyte aging that influenced the subsequent *in vitro* development of the intraspSCNT embryos. In conjunction to this, screening of the suitable IVM duration for LOPU and abattoir-derived oocyte was done and LOPU oocyte seems to mature at a shorter IVM duration and the IVD competency of the intraspSCNT embryo improved in the subsequent intraspSCNT experiments. However, it is worth mentioning that beside the IVM duration, the possibility of deficiency in skills acquired at the early phase of the research might influence the IVD competency of the early caprine intraspSCNT study as well.

d) Embryo developmental arrest:

One of the common and major problems faced by all the researchers in this laboratory was to obtain *in vitro* produced (via IVF, ICSI and SCNT) caprine blastocysts in their studies. The embryo development was unfailingly blocked at morula stage when the previous IVC system using mSOFaa was used. This frustrated scenario had led to the discovery of a new two-step IVC system using KSOMaa as basal medium that could support the development of caprine cloned blastocysts after a series of effort invested to troubleshoot other factors that was speculated to influence the blastocyst development as well such as IVM medium, duration, activation treatment, substitution of BSA with FBS in mSOFaa, among other factors.

e) Evaluation of blastocyst quality:

One of the loop holes in this aspect was the failure to assess the quality of blastocyst in terms of the ratio of TE: ICM using the differential staining approach. Our laboratory had the technical limitation in producing the rabbit anti-goat spleenocyte antibodies and therefore, this method of staining could not be carried out in this study instead was substituted by the normal Hoechst 33342 staining.

f) Facilities and man-power:

It is worth to note that, the facilities and equipment available in the present laboratory was insufficient to enable this SCNT research to be carried out in a more comprehensive manner. Sharing of equipment, particularly the micromanipulator with other researchers who run their experiment concurrently do not permits SCNT experiment in each replicate to be carried out at the exact time point and this might influence slightly the outcome of the research. As retrieval of oocyte via LOPU require the assistance of several personnel, this poses challenges for the OR procedure if insufficient man-power was encountered. The author was fortunately to obtain the assistance from both the project supervisors and other laboratory members to overcome this hurdle during the OR procedure.

Even though, there were some loop holes in this study, the overall findings of the present study formed a basis for more detailed studies to improve the protocol for the production of cloned embryos and kids via intraspSCNT and interspSCNT approaches. The suggested aspects for future studies encompass:

a) Using cloned caprine blastocyst as a bringing step for embryonic stem cell research.
b) Application of this study to increase animal production, alleviation in the production of endangered animal using interspSCNT.
c) Optimisation of donor karyoplast factors such as the different somatic cell type, cell cycle coordination and number of passage
d) Treatment of donor cells with chromatin remodeling prior nuclear transfer to facilitate nuclear reprogramming of the reconstructed caprine embryos.
e) Optimisation of time interval between fusion and activation for caprine reconstructed oocytes using skin fibroblast cell as donor karyoplast.
f) Improvement in the evaluation of blastocyst quality using assessment by differential counting of inner cell mass (ICM) and trophectoderm (TE) cells and by apoptosis detection in blastomeres using a terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling (TUNEL) assay.
g) Detailed study on the relevant biological question such as the mechanism behind the nuclear reprogramming, zygotic genome activation (ZGA) or how to overcome the issue of mitochondria genomic DNA composition incompatibility between oocyte and nuclei donors of large phylogenetic distance, particularly on the caprine- bovine interspSCNT.
h) Improvement on the embryo transfer efficiency by increasing the number of cloned embryos transferred per recipient.

Chapter 6

6.0 CONCLUSIONS

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This study presents the development of cloned caprine embryos produced through (caprine-caprine) intraspecies somatic cell nuclear transfer (intraspSCNT) and (caprine-bovine) interspecies somatic cell nuclear transfer (interspSCNT) techniques using caprine and bovine oocytes as recipient cytoplasts, respectively. Caprine ear skin fibroblast cells were used as donor karyoplasts. From the findings obtained in this study, it can be concluded that:

a) Cloned caprine embryos could be produced successfully up to blastocyst stage using both intraspSCNT and interspSCNT approaches in the local setting of Animal Biotechnology-Embryo Laboratory, University of Malaya.
b) Both pregnant mare's serum gonadotrophin (PMSG) and porcine-derived follicle stimulating hormone (pFSH) employed in the designated regimes have relatively similar potential to stimulate caprine ovaries for oocyte retrieval via laparoscopic ovum pick-up (LOPU). However, the efficacy of PMSG could not surpass the pFSH, particularly, when it was employed in the repeated ovarian stimulation and oocyte retrieval programme. [Objective (a)]
c) Oocyte retrieval from LOPU source produced better quality oocytes (Grades A and B) even though the oocyte yield was lower compared to abattoir source. Correspondingly, caprine oocytes from LOPU gave higher maturation rate than abattoir-derived ovaries. [Objective (b)]
d) The optimum *in vitro* maturation (IVM) duration for caprine oocytes retrieved from LOPU- and abattoir derived-ovaries in this laboratory setting were 18 to 22 hours and 22 to 26 hours, respectively. [Objective (c)]

e) Both cloned bovine and gaur embryos could be produced *in vitro* up to hatched blastocyst stage; suggesting that the manipulation technique and protocol could be used as a reference for the caprine SCNT studies. [Objective (d)]

f) Caprine oocytes from superstimulated does optimally matured at 18 to 22 hours gave a significantly higher maturation rate, enucleation rate and *in vitro* developmental (IVD) rates (2-cell to morula stage) than oocytes matured at 23 to 27 hours after intraspSCNT. [Objective (e)]

g) Both activation protocols [(7% EtOH + CD-CHX) and (CaI + 6-DMAP)] had comparable efficiency in inducing the development of caprine reconstructed embryos. [Objective (f)]

h) Potassium simplex optimisation medium with amino acid (KSOMaa) basal medium supported the *in vitro* development of cloned caprine embryos better than modified synthetic oviduct fluid with amino acid (mSOFaa) in the one-step culture system, as cloned blastocyst could only be developed when cultured in KSOMaa in this study. [Objective (g)]

i) Increasing glucose supplementation to 2.78 mM in KSOMaa medium at Day 2 of IVC enhanced the cloned caprine blastocyst rate and promoted hatching of blastocyst. [Objective (h)]

j) Both intraspSCNT and interspSCNT (caprine karyoplast-bovine cytoplast) approaches could be used to produce cloned caprine blastocyst, however, the efficiency of interspSCNT approach was still low compared to the intraspSCNT approach. [Objective (i)]

k) No pregnancy was detected after the attempt of embryo transfer on the cloned caprine embryos. [Objective (i)]

l) In summary, cloned caprine embryos could be produced successfully up to blastocyst stage using ear skin fibroblast cell via intraspSCNT and interspSCNT approaches by incorporating the following summarised protocols:

Table 6.1: A summary of the proposed protocol for the development of caprine intraspSCNT and interspSCNT embryos

Aspect	intraspSCNT		interspSCNT
Source of recipient oocytes	LOPU-derived caprine oocytes	Abattoir-derived caprine oocytes	Abattoir-derived bovine oocytes
Source of gonadotrophins for repeated ovarian stimulation and oocyte retrieval programme	pFSH regime	Not applicable	Not applicable
IVM duration	18 to 22 hours	22 to 26 hours	22 to 24 hours
Donor karyoplast	Caprine ear skin fibroblast cell (Passages 3 to 5)		
Activation treatment	CaI [5 µM] (5 min) followed with 6-DMAP [2 mM] (4 h)		
IVC system	Two-step culture system: Day 0-2 (post-activation): KSOMaa Day 2-8: KSOMaa + glucose (2.78 mM) final concentration Incubation: 38.5°C under a humidified atmosphere of 5% CO_2 in air		

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APPENDICES

APPENDICES

APPENDIX 1: LIST OF MATERIALS

Appendix Table 1.1: List of equipment and instruments

Equipment/instrument	Model no.	Manufacturer
Abrasive stone or oilstone		Hall's Arkansas Oilstones, USA
Atraumatic grasping forceps	PO951R	Aesculap ®, Germany
Autoclave	HA-300MII	Hirayama Hiclave, Japan
Centrifuge	D37520	Heraeus, Germany
CIDR applicator	-	Pharmacia and Upjohn, New Zealand
CO_2 incubator	HeraCell 240	Heraeus,Germany
CO_2 insufflator system	PG001	Aesculap® , Germany
Digital balance	AB104	Mettler Toledo, Switzerland
Digital camera (X-Cam-α)	-	microLAMBDA Sdn Bhd, Malaysia
Dissecting microscope	SZH10	Olympus, Japan
Electrofusion machine	SUTF-1	Suranaree University of Technology, Thailand
Flushing and aspiration system:		
(a) Aspiration system	KMAR-5100	Cook, Australia
(b) Flushing system	KMAR-4000	Cook, Australia
(c)Test tube heater system	KFTH-1012	Cook, Australia
(d) Pedal	6210-725350B	Herga Electric Ltd, UK
Heating stage (Thermoplate)	HATS-U55R30	Tokai Hit, Japan
Impulse sealer	KF-300H	Khind, Taiwan
Inverted microscope	IX71	Olympus, Japan
Laminar flow cabinet	HLF-120	Gelman Sciences, Australia
Laparoscopic system :		Aesculap® , Germany
(a) Endoscopic camera system	PV431	
(b) CCD camera	PV430	
(c) Pediatric Storz laparoscope(7mm)	PE688A	
(d) Light probe with fibre optic cable	OP913	
(e) Light system (300W)	OP927	
Liquid nitrogen tank (small)	SC2/IV	MVE, USA
Microforge	-	Technical Products Internationals, USA
Microgrinder	EG-4	Narishige, Japan

(continued)

Equipment/instrument	Model no.	Manufacturer
Micropipette dispenser	-	Eppendorf, Germany
Micropipette puller	P-97	Sutter Instrument Co, USA
Narishige hydraulic micromanipulators	ON3-99D	Narishige, Japan
Oocyte pick-up needle	FAS set C2	Gynetics Medical Product, Belgium
Osmometer	Vapro 5520	WESCOR Inc., USA
Oven	40050-IP20	Memmert GmbH, Germany
pH meter	HI-122	Hanna Instruments, Singapore
Pipette pump	PI-PUMP	Glasfirn, Germany
Refrigerator and freezer	SR-21NME	Samsung Electronics, Korea
Spirit burner	-	Shanghai Machinery, China
Stereomicroscope	SZH10	Olympus Optical, Japan
Surgical set	-	Aesculap® , Germany
Surgical table	-	Syarikat Copens Enterprise,Malaysia
Trocar and canula (5.5 mm & 7.0 mm)	EJ456, EJ457	Aesculap® , Germany
Ultrapure water purification system	Milli-Q PF Plus	Millipore, USA
Vapour pressure osmometer	5520	Vapro Wescor, USA
Verrus needle	PG3	Cook, Australia
Vortex mixer	VTX-3000L	LMS, Japan
Water bath	GMP-GC-19	Memmert GmbH, Germany

Appendix Table 1.2: List of chemicals, reagents and media

Chemicals, reagents and media	Catalogue no.	Manufacturer
6-dimethylaminopurine (6-DMAP)	D2629	Sigma-Aldrich, USA
70% ethanol	-	Prepared from absolute ethanol
α- Minimum Essential Medium Eagle	M0644	
Ethyl alcohol 99.8% (absolute ethanol)	ET150-50	Systerm ChemAR® , Poland
Hibiscrub (antiseptic)	HK-06770	SSL International Plc, UK
BME amino acids solution (50X)	B6766	Sigma-Aldrich, USA
Bovine serum albumin fraction V (BSA-V)	A6003	Sigma-Aldrich, USA
Calcium acetate hydrate ($Ca(C_2H_3O_2)_2.xH_2O$)	C4705	Sigma-Aldrich, USA

(continued)

Chemicals, reagents and media	Catalogue no.	Manufacturer
Calcium chloride ($CaCl_2$)	C5670	Sigma-Aldrich, USA
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	C7902	Sigma-Aldrich, USA
Cleaning solution 7X® -PF	-	FlowLab™ , Australia
Cloprostenol (Estrumate®)	-	Schering-Plough, Australia
Cycloheximide (CHX)	C7698	Sigma-Aldrich,USA
Cysteamine	M9768	Sigma-Aldrich, USA
Cytochalasin B ($C_{29}H_{37}NO_5$)	C6762	Sigma-Aldrich, USA
Cytochalasin D ($C_{30}H_{37}NO_6$)	C8273	Sigma-Aldrich, USA
D-(+)-Glucose ($C_6H_{12}O_6$)	G6152	Sigma-Aldrich, USA
Dimethyl sulphoxide (DMSO)	D5879	Sigma-Aldrich, USA
Disinfectant Gigasept FF	-	Schulke&Mary GmbH, Germany
Ethylenediaminetetraacetic acid (EDTA)	E9884	Sigma-Aldrich,USA
EMCARE ™	ECHM-500	ICPbio Reproduction, NZ
Foetal Bovine Serum (FBS)	16000-044	Gibco BRL, USA
Formaldehyde solution (40%)	F8775	Sigma-Aldrich, USA
FSH (pFSH)	Folltropin-V®	Bioniche, NZ
Gentamicin sulfate salt	G3632	Sigma-Aldrich, USA
Goat/sheep pellet feed	-	KMM Berhad, Malaysia
Glutaraldehyde solution (25%)	G5882	Sigma-Aldrich, USA
Glycerol	G2025	Sigma-Aldrich, USA
Heparin	H0777	Sigma-Aldrich, USA
HEPES	H7006	Sigma-Aldrich, USA
Hoechst 33342	B2261	Sigma-Aldrich, USA
Hyaluronidase (from bovine testes)	H4272	Sigma-Aldrich, USA
Hydrochloric acid	HY450-70	Systerm ChemAR® , Poland
Hydrofluoric acid	1301030	HmbG Chemicals, Germany
Intravaginal progesterone release device	-	Pharmacia and Upjohn, New Zealand
Ketamil injection (ketamine hydrochloride)	L100771	Troy Laboratories, Australia
K-Y Lubricating Jelly	-	Pharmedica Lab, South Africa
L-Cysteine hydrochloride	C7477	Sigma-Aldrich, USA
L-Glutamine	G3126	Sigma-Aldrich, USA
L-Glutathione reduced	G4251	Sigma-Aldrich, USA
Liquid nitrogen	-	Mox Gases Berhad, Malaysia
Magnesium acetate tetrahydrate (Mg(C2H3O2)2.4H2O)	M0631	Sigma-Aldrich, USA
Magnesium chloride hexahydrate (MgCl2.6H2O)	M2393	Sigma-Aldrich, USA

(continued)

Chemicals, reagents and media	Catalogue no.	Manufacturer
Magnesium sulphate ($MgSO_4$)	M7506	Sigma-Aldrich, USA
MEM non-essential amino acids solution [100x]	M7145	Sigma-Aldrich, USA
Mineral oil	M8410	Sigma-Aldrich, USA
Oestradiol-17β	E4389	Sigma-Aldrich, USA
Ovidrel® PreFilled Syringe	-	Laboratories Serono, Switzerland
Oxytetracycline (Tetrasol 20%)	E388	Richter Pharma, Austria
Penicillin G sodium salt	P7794	Sigma-Aldrich,USA
PBS Dulbecco A tablets	BR0014G	Oxoid, England
Phenol red (0.5%)	P0290	Sigma-Aldrich,USA
PMSG (eCG)	-	Intervet International, Holland
Polyvinylpyrrolidone-360	PVP360	Sigma-Aldrich,USA
Potassium chloride (KCl)	P5405	Sigma-Aldrich,USA
Potassium phosphate dibasic (K_2HPO_4)	P3786	Sigma-Aldrich,USA
Potassium phosphate monobasic (KH_2PO_4)	P5655	Sigma-Aldrich,USA
Sodium bicarbonate ($NaHCO_3$)	S5761	Sigma-Aldrich,USA
Sodium chloride (NaCl)	S5886	Sigma-Aldrich,USA
Sodium DL-lactate	L7900	Sigma-Aldrich,USA
Sodium phosphate dibasic (Na_2HPO_4)	S5136	Sigma-Aldrich,USA
Sodium pyruvate	P4562	Sigma-Aldrich,USA
Streptomycin sulfate salt	S1277	Sigma-Aldrich,USA
Sucrose ($C_{12}H_{22}O_{11}$)	S7903	Sigma-Aldrich,USA
TCM-199	M4530	Sigma-Aldrich,USA
Trypsin from porcine pancreas	T4799	Sigma-Aldrich,USA
Weak iodine solution	-	ICN Biomedicals, USA
Xylazine hydrochloride (Ilium Xylazil-20)	L10600	Troy Laboratories, Australia

Appendix Table 1.3: List of labwares and disposables

Labwares and disposables	Manufacturer
Aluminium foil	Reynolds Consumer Products, USA
Autoclave disposal bag	Megalab supplies, Malaysia
Blades (Super Nacet)	Gillette, USA
Borosilicate glass tubing (Microcaps®)	Drummond Scientific Company, USA
Chromic catgut and other suture materials	Aesculap®, Germany
Culture dish	Nunc, Denmark
Disposable glass Pasteur pipette	Hirschmann® Laborgerete, Germany
Disposable hand tissues	Megalab supplies, Malaysia
Falcon™ conical tube	Becton Dickinson, USA
Falcon™ polystyrene round-bottom test tube	Becton Dickinson, USA
Glassware (beaker, flask, measuring cylinder etc.)	Pyrex®, Japan
Lens cleansing tissue (Kimswipe® EX-L)	Kimberly-Clark, USA
Microcentrifuge tube	Elkay, Costelloe
Micropipette tips without filter	Axygen Scientific, USA
Microscope slide	Sail Brand, China
Microscope glass cover slip	Hirschmann® Laborgerete, Germany
Millex® –GS syringe driven filter	Schleicher and Schuell, Germany
Needle	Terumo Corporation, Japan
Parafilm	Pechiney Plastic Packaging, USA
Schott bottle	Duran, Germany
Serological pipette	LP Italiana SPA, Italy
Sterile glove	Ansell International, Malaysia
Syringe	Terumo Corporation, Japan
Tissue culture flask	Nunc, Denmark
Terumo venojector holder	Terumo Corporation, Japan
Vacutainer ® blood collection tubes	Becton Dickinson, USA
Vacutainer needle	Becton Dickinson, USA

APPENDIX 2: STATISTICAL DATA

Appendix Table 2.1: Number and percentages of oocytes retrieved from caprine superstimulated with PMSG according to OR cycle (Experiment 1)

Descriptives

Goat superstimulated with PMSG

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Number of follicle	OR1	36	8.8333	3.08452	.51409	7.7897	9.8770	4.00	15.00
	OR2	18	8.0000	2.42536	.57166	6.7939	9.2061	4.00	12.00
	OR3	6	4.8333	1.94079	.79232	2.7966	6.8701	3.00	8.00
	Total	60	8.1833	3.01123	.38875	7.4054	8.9612	3.00	15.00
Number of oocyte retrieved per ovary	OR1	36	5.6111	3.53172	.58862	4.4162	6.8061	1.00	15.00
	OR2	18	6.0556	2.20887	.52063	4.9571	7.1540	3.00	11.00
	OR3	6	3.1667	.75277	.30732	2.3767	3.9567	2.00	4.00
	Total	60	5.5000	3.08358	.39809	4.7034	6.2966	1.00	15.00
Oocyte retrieval rate upon number of follicle predicted	OR1	36	60.7083	25.66365	4.27728	52.0250	69.3917	16.67	100.00
	OR2	18	76.7889	18.10426	4.26722	67.7859	85.7919	37.50	100.00
	OR3	6	72.3617	25.82198	10.54178	45.2632	99.4602	37.50	100.00
	Total	60	66.6978	24.45146	3.15667	60.3814	73.0143	16.67	100.00

ANOVA

Goat superstimulated with PMSG

		Sum of Squares	df	Mean Square	F	Sig.
Number of follicle	Between Groups	83.150	2	41.575	5.245	.008
	Within Groups	451.833	57	7.927		
	Total	534.983	59			
Number of oocyte retrieved per ovary	Between Groups	38.667	2	19.333	2.110	.131
	Within Groups	522.333	57	9.164		
	Total	561.000	59			
Oocyte retrieval rate upon number of follicle predicted	Between Groups	3316.871	2	1658.436	2.958	.060
	Within Groups	31957.676	57	560.661		
	Total	35274.548	59			

Post Hoc Tests

Homogeneous Subsets

Number of follicle for goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05	
		1	2
OR3	6	4.8333	
OR2	18		8.0000
OR1	36		8.8333
Sig.		1.000	.471

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Number of oocyte retrieved per ovary for goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05	
		1	2
OR3	6	3.1667	
OR1	36	5.6111	5.6111
OR2	18		6.0556
Sig.		.053	.720

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Oocyte retrieval rate upon number of follicle predicted for goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05
		1
OR1	36	60.7083
OR3	6	72.3617
OR2	18	76.7889
Sig.		.121

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix Table 2.2: Percentages of oocytes retrieved from caprine superstimulated with PMSG among 3 OR cycles within each oocyte grade (Experiment 1)

Descriptives

Goat superstimulated with PMSG

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Grade A	OR1	36	29.3019	27.49408	4.58235	19.9993	38.6046	.00	100.00
	OR2	18	28.4850	18.19354	4.28826	19.4376	37.5324	.00	62.50
	OR3	6	19.4450	26.17724	10.68682	-8.0263	46.9163	.00	66.67
	Total	60	28.0712	24.70667	3.18962	21.6888	34.4536	.00	100.00
Grade B	OR1	36	28.1681	30.41431	5.06905	17.8773	38.4588	.00	100.00
	OR2	18	29.4867	17.20109	4.05434	20.9328	38.0406	.00	60.00
	OR3	6	16.6650	18.25559	7.45281	-2.4931	35.8231	.00	33.33
	Total	60	27.4133	25.99327	3.35572	20.6986	34.1281	.00	100.00
Grade C	OR1	36	32.2547	27.23684	4.53947	23.0391	41.4703	.00	100.00
	OR2	18	22.2489	20.45400	4.82105	12.0774	32.4204	.00	62.50
	OR3	6	26.3883	22.61780	9.23368	2.6524	50.1243	.00	50.00
	Total	60	28.6663	24.99852	3.22730	22.2085	35.1241	.00	100.00
Grade D	OR1	36	8.8847	13.16613	2.19436	4.4299	13.3395	.00	50.00
	OR2	18	11.5367	14.81146	3.49109	4.1711	18.9022	.00	50.00
	OR3	6	23.6117	29.06828	11.86708	-6.8936	54.1170	.00	66.67
	Total	60	11.1530	16.01937	2.06809	7.0148	15.2912	.00	66.67
Grade E	OR1	36	1.3889	8.33333	1.38889	-1.4307	4.2085	.00	50.00
	OR2	18	8.2411	17.02125	4.01195	-.2234	16.7056	.00	66.67
	OR3	6	13.8883	22.15208	9.04355	-9.3589	37.1355	.00	50.00
	Total	60	4.6945	13.61478	1.75766	1.1774	8.2116	.00	66.67

ANOVA

Goat superstimulated with PMSG

		Sum of Squares	df	Mean Square	F	Sig.
Grade A	Between Groups	504.080	2	252.040	.405	.669
	Within Groups	35510.680	57	622.994		
	Total	36014.761	59			
Grade B	Between Groups	791.043	2	395.521	.577	.565
	Within Groups	39072.307	57	685.479		
	Total	39863.349	59			
Grade C	Between Groups	1235.996	2	617.998	.989	.378
	Within Groups	35634.640	57	625.169		
	Total	36870.636	59			
Grade D	Between Groups	1119.183	2	559.591	2.275	.112
	Within Groups	14021.418	57	245.990		
	Total	15140.601	59			
Grade E	Between Groups	1126.946	2	563.473	3.274	.045
	Within Groups	9809.421	57	172.095		
	Total	10936.367	59			

Post Hoc Tests

Homogeneous Subsets

Grade A retrieved from goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05
		1
OR3	6	19.4450
OR2	18	28.4850
OR1	36	29.3019
Sig.		.368

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade B retrieved from goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05
		1
OR3	6	16.6650
OR1	36	28.1681
OR2	18	29.4867
Sig.		.264

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade C retrieved from goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05
		1
OR2	18	22.2489
OR3	6	26.3883
OR1	36	32.2547
Sig.		.361

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade D retrieved from goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05	
		1	2
OR1	36	8.8847	
OR2	18	11.5367	11.5367
OR3	6		23.6117
Sig.		.680	.064

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade E retrieved from goat superstimulated with PMSG

Duncan[a,,b]

	N	Subset for alpha = 0.05	
		1	2
OR1	36	1.3889	
OR2	18	8.2411	8.2411
OR3	6		13.8883
Sig.		.206	.296

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 12.000.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix Table 2.3: Percentages of oocytes retrieved from caprine superstimulated with PMSG among oocyte grades within each OR cycle (Experiment 1)

Goat superstimulated with PMSG

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	Grade A	36	29.30194	27.494080	4.582347	19.99929	38.60460	.000	100.000
	Grade B	36	28.16806	30.414307	5.069051	17.87733	38.45878	.000	100.000
	Grade C	36	32.25472	27.236840	4.539473	23.03910	41.47034	.000	100.000
	Grade D	36	8.88472	13.166133	2.194356	4.42994	13.33950	.000	50.000
	Grade E	36	1.38889	8.333333	1.388889	-1.43071	4.20848	.000	50.000
	Total	180	19.99967	26.014188	1.938983	16.17346	23.82587	.000	100.000
OR2	Grade A	18	28.48500	18.193542	4.288259	19.43756	37.53244	.000	62.500
	Grade B	18	29.48667	17.201095	4.054337	20.93276	38.04057	.000	60.000
	Grade C	18	22.24889	20.454003	4.821055	12.07735	32.42043	.000	62.500
	Grade D	18	11.53667	14.811455	3.491094	4.17110	18.90223	.000	50.000
	Grade E	18	8.24111	17.021252	4.011948	-.22336	16.70558	.000	66.670
	Total	90	19.99967	19.316713	2.036160	15.95386	24.04547	.000	66.670
OR3	Grade A	6	19.44500	26.177245	10.686815	-8.02633	46.91633	.000	66.670
	Grade B	6	16.66500	18.255593	7.452815	-2.49307	35.82307	.000	33.330
	Grade C	6	26.38833	22.617799	9.233678	2.65241	50.12426	.000	50.000
	Grade D	6	23.61167	29.068279	11.867075	-6.89362	54.11695	.000	66.670
	Grade E	6	13.88833	22.152084	9.043550	-9.35885	37.13552	.000	50.000
	Total	30	19.99967	22.700921	4.144602	11.52300	28.47633	.000	66.670

Goat superstimulated with PMSG

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	27840.398	4	6960.099	13.055	.000
	Within Groups	93295.701	175	533.118		
	Total	121136.099	179			
OR2	Between Groups	6785.083	4	1696.271	5.457	.001
	Within Groups	26423.968	85	310.870		
	Total	33209.051	89			
OR3	Between Groups	615.826	4	153.956	.269	.895
	Within Groups	14328.796	25	573.152		
	Total	14944.622	29			

Post Hoc Tests

Homogeneous Subsets

OR1

Duncan[a]

		Subset for alpha = 0.05	
Treatment	N	1	2
Grade E	36	1.38889	
Grade D	36	8.88472	
Grade B	36		28.16806
Grade A	36		29.30194
Grade C	36		32.25472
Sig.		.170	.484

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 36.000.

OR2

Duncan[a]

		Subset for alpha = 0.05		
Treatment	N	1	2	3
Grade E	18	8.24111		
Grade D	18	11.53667	11.53667	
Grade C	18		22.24889	22.24889
Grade A	18			28.48500
Grade B	18			29.48667
Sig.		.576	.072	.250

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 18.000.

OR3

Duncan[a]

		Subset for alpha = 0.05
Treatment	N	1
Grade E	6	13.88833
Grade B	6	16.66500
Grade A	6	19.44500
Grade D	6	23.61167
Grade C	6	26.38833
Sig.		.427

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix Table 2.4: Number and percentages of oocytes retrieved from caprine superstimulated with pFSH according to OR cycle (Experiment 1)

Descriptives

Goat superstimulated with pFSH

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
No of follicle	OR1	30	8.20000	2.975764	.543298	7.08883	9.31117	4.000	15.000
	OR2	10	7.60000	2.270585	.718022	5.97572	9.22428	5.000	12.000
	OR3	4	7.00000	1.825742	.912871	4.09484	9.90516	5.000	9.000
	Total	44	7.95455	2.727590	.411200	7.12528	8.78381	4.000	15.000
No of oocyte	OR1	30	6.23333	3.191404	.582668	5.04164	7.42502	2.000	15.000
	OR2	10	5.70000	1.636392	.517472	4.52940	6.87060	3.000	8.000
	OR3	4	5.75000	1.500000	.750000	3.36317	8.13683	4.000	7.000
	Total	44	6.06818	2.765214	.416872	5.22748	6.90888	2.000	15.000
Retrieval rate	OR1	30	74.27967	22.438950	4.096773	65.90083	82.65851	33.330	100.000
	OR2	10	76.44800	16.374905	5.178200	64.73410	88.16190	58.330	100.000
	OR3	4	82.98750	14.179406	7.089703	60.42490	105.55010	66.670	100.000
	Total	44	75.56409	20.400539	3.075497	69.36176	81.76642	33.330	100.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
No of follicle	Between Groups	6.709	2	3.355	.439	.648
	Within Groups	313.200	41	7.639		
	Total	319.909	43			
No of oocyte	Between Groups	2.579	2	1.289	.162	.851
	Within Groups	326.217	41	7.957		
	Total	328.795	43			
Retrieval rate	Between Groups	277.733	2	138.867	.323	.726
	Within Groups	17618.092	41	429.710		
	Total	17895.825	43			

Post Hoc Tests

Homogeneous Subsets

No of follicle

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR3	4	7.00000
OR2	10	7.60000
OR1	30	8.20000
Sig.		.425

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

No of oocyte

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR2	10	5.70000
OR3	4	5.75000
OR1	30	6.23333
Sig.		.728

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Oocyte retrieval rate

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR1	30	74.27967
OR2	10	76.44800
OR3	4	82.98750
Sig.		.440

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix Table 2.5: Percentages of oocytes retrieved from caprine superstimulated with PMSG among 3 OR cycles within each oocyte grade (Experiment 1)

Descriptives

Goat superstimulated with pFSH

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Grade A	OR1	30	36.23033	21.752476	3.971441	28.10783	44.35284	.000	80.000
	OR2	10	32.50000	28.360336	8.968326	12.21224	52.78776	.000	100.000
	OR3	4	41.25000	13.174587	6.587293	20.28629	62.21371	25.000	57.140
	Total	44	35.83886	22.472009	3.387783	29.00675	42.67098	.000	100.000
Grade B	OR1	30	41.37767	20.713003	3.781660	33.64330	49.11203	.000	100.000
	OR2	10	20.02400	17.027847	5.384678	7.84301	32.20499	.000	50.000
	OR3	4	26.78750	23.600163	11.800081	-10.76563	64.34063	.000	50.000
	Total	44	35.19818	21.809617	3.287924	28.56745	41.82891	.000	100.000
Grade C	OR1	30	16.44200	19.566429	3.572325	9.13578	23.74822	.000	66.670
	OR2	10	25.11900	19.331112	6.113034	11.29036	38.94764	.000	50.000
	OR3	4	15.71500	12.008387	6.004194	-3.39302	34.82302	.000	28.570
	Total	44	18.34795	18.981950	2.861637	12.57691	24.11899	.000	66.670
Grade D	OR1	30	5.18000	8.645736	1.578488	1.95163	8.40837	.000	25.000
	OR2	10	16.52400	15.639011	4.945490	5.33653	27.71147	.000	50.000
	OR3	4	16.25000	19.737865	9.868933	-15.15735	47.65735	.000	40.000
	Total	44	8.76455	12.528442	1.888734	4.95555	12.57354	.000	50.000
Grade E	OR1	30	.80300	2.471644	.451258	-.11993	1.72593	.000	9.090
	OR2	10	5.83300	12.452800	3.937921	-3.07520	14.74120	.000	33.330
	OR3	4	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	44	1.87318	6.430319	.969407	-.08181	3.82818	.000	33.330

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Grade A	Between Groups	233.199	2	116.600	.223	.801
	Within Groups	21481.423	41	523.937		
	Total	21714.622	43			
Grade B	Between Groups	3731.097	2	1865.549	4.574	.016
	Within Groups	16722.257	41	407.860		
	Total	20453.355	43			
Grade C	Between Groups	595.180	2	297.590	.819	.448
	Within Groups	14898.340	41	363.374		
	Total	15493.521	43			
Grade D	Between Groups	1211.688	2	605.844	4.486	.017
	Within Groups	5537.672	41	135.065		
	Total	6749.360	43			
Grade E	Between Groups	205.196	2	102.598	2.675	.081
	Within Groups	1572.812	41	38.361		
	Total	1778.007	43			

Post Hoc Tests

Homogeneous Subsets

Grade A

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR2	10	32.50000
OR1	30	36.23033
OR3	4	41.25000
Sig.		.482

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade B

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR2	10	20.02400
OR3	4	26.78750
OR1	30	41.37767
Sig.		.053

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade C

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR3	4	15.71500
OR1	30	16.44200
OR2	10	25.11900
Sig.		.364

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade D

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR1	30	5.18000
OR3	4	16.25000
OR2	10	16.52400
Sig.		.074

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Grade E

Duncan[a,,b]

Treatment	N	Subset for alpha = 0.05
		1
OR3	4	.00000
OR1	30	.80300
OR2	10	5.83300
Sig.		.085

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.826.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

Appendix Table 2.6: Percentages of oocytes retrieved from caprine superstimulated with PMSG among oocyte grades within each OR cycle (Experiment 1)

Descriptives

Goat superstimulated with pFSH

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	Grade A	30	36.23033	21.752476	3.971441	28.10783	44.35284	.000	80.000
	Grade B	30	41.37767	20.713003	3.781660	33.64330	49.11203	.000	100.000
	Grade C	30	16.44200	19.566429	3.572325	9.13578	23.74822	.000	66.670
	Grade D	30	5.18000	8.645736	1.578488	1.95163	8.40837	.000	25.000
	Grade E	30	.80300	2.471644	.451258	-.11993	1.72593	.000	9.090
	Total	150	20.00660	23.062286	1.883028	16.28571	23.72749	.000	100.000
OR2	Grade A	10	32.50000	28.360336	8.968326	12.21224	52.78776	.000	100.000
	Grade B	10	20.02400	17.027847	5.384678	7.84301	32.20499	.000	50.000
	Grade C	10	25.11900	19.331112	6.113034	11.29036	38.94764	.000	50.000
	Grade D	10	16.52400	15.639011	4.945490	5.33653	27.71147	.000	50.000
	Grade E	10	5.83300	12.452800	3.937921	-3.07520	14.74120	.000	33.330
	Total	50	20.00000	20.583728	2.910979	14.15017	25.84983	.000	100.000
OR3	Grade A	4	41.25000	13.174587	6.587293	20.28629	62.21371	25.000	57.140
	Grade B	4	26.78750	23.600163	11.800081	-10.76563	64.34063	.000	50.000
	Grade C	4	15.71500	12.008387	6.004194	-3.39302	34.82302	.000	28.570
	Grade D	4	16.25000	19.737865	9.868933	-15.15735	47.65735	.000	40.000
	Grade E	4	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	20	20.00050	19.885451	4.446522	10.69382	29.30718	.000	57.140

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	39637.341	4	9909.335	36.274	.000
	Within Groups	39611.147	145	273.180		
	Total	79248.488	149			
OR2	Between Groups	3952.412	4	988.103	2.645	.046
	Within Groups	16808.391	45	373.520		
	Total	20760.803	49			
OR3	Between Groups	3720.226	4	930.056	3.678	.028
	Within Groups	3792.966	15	252.864		
	Total	7513.192	19			

Post Hoc Tests

Homogeneous Subsets

OR1

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Grade E	30	.80300		
Grade D	30	5.18000		
Grade C	30		16.44200	
Grade A	30			36.23033
Grade B	30			41.37767
Sig.		.307	1.000	.230

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

OR2

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
Grade E	10	5.83300	
Grade D	10	16.52400	16.52400
Grade B	10	20.02400	20.02400
Grade C	10		25.11900
Grade A	10		32.50000
Sig.		.128	.097

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 10.000.

OR3

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
Grade E	4	.00000	
Grade C	4	15.71500	15.71500
Grade D	4	16.25000	16.25000
Grade B	4		26.78750
Grade A	4		41.25000
Sig.		.190	.052

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Appendix Table 2.7: Number of follicle recruited from does superstimulated with PMSG vs. pFSH within each OR cycle (Experiment 1)

Descriptives

No of follicle

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	PMSG	36	8.83333	3.084524	.514087	7.78968	9.87699	4.000	15.000
	pFSH	30	8.20000	2.975764	.543298	7.08883	9.31117	4.000	15.000
	Total	66	8.54545	3.028997	.372844	7.80083	9.29008	4.000	15.000
OR2	PMSG	18	8.00000	2.425356	.571662	6.79390	9.20610	4.000	12.000
	pFSH	10	7.60000	2.270585	.718022	5.97572	9.22428	5.000	12.000
	Total	28	7.85714	2.336732	.441601	6.95105	8.76323	4.000	12.000
OR3	PMSG	6	4.83333	1.940790	.792324	2.79660	6.87007	3.000	8.000
	pFSH	4	7.00000	1.825742	.912871	4.09484	9.90516	5.000	9.000
	Total	10	5.70000	2.110819	.667499	4.19001	7.20999	3.000	9.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	6.564	1	6.564	.712	.402
	Within Groups	589.800	64	9.216		
	Total	596.364	65			
OR2	Between Groups	1.029	1	1.029	.183	.673
	Within Groups	146.400	26	5.631		
	Total	147.429	27			
OR3	Between Groups	11.267	1	11.267	3.126	.115
	Within Groups	28.833	8	3.604		
	Total	40.100	9			

Appendix Table 2.8: Number of oocyte retrieved from does superstimulated with PMSG vs. pFSH within each OR cycle (Experiment 1)

Descriptives

No of oocyte

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	PMSG	36	5.61111	3.531716	.588619	4.41615	6.80607	1.000	15.000
	pFSH	30	6.23333	3.191404	.582668	5.04164	7.42502	2.000	15.000
	Total	66	5.89394	3.370135	.414835	5.06546	6.72242	1.000	15.000
OR2	PMSG	18	6.05556	2.208865	.520634	4.95711	7.15400	3.000	11.000
	pFSH	10	5.70000	1.636392	.517472	4.52940	6.87060	3.000	8.000
	Total	28	5.92857	1.998677	.377714	5.15357	6.70358	3.000	11.000
OR3	PMSG	6	3.16667	.752773	.307318	2.37668	3.95665	2.000	4.000
	pFSH	4	5.75000	1.500000	.750000	3.36317	8.13683	4.000	7.000
	Total	10	4.20000	1.686548	.533333	2.99352	5.40648	2.000	7.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	6.335	1	6.335	.554	.459
	Within Groups	731.922	64	11.436		
	Total	738.258	65			
OR2	Between Groups	.813	1	.813	.197	.661
	Within Groups	107.044	26	4.117		
	Total	107.857	27			
OR3	Between Groups	16.017	1	16.017	13.370	.006
	Within Groups	9.583	8	1.198		
	Total	25.600	9			

Appendix Table 2.9: Percentage of Grade A oocyte retrieved from does superstimulated with PMSG vs. pFSH within each OR cycle (Experiment 1)

Descriptives

Grade A

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	PMSG	36	29.30194	27.494080	4.582347	19.99929	38.60460	.000	100.000
	pFSH	30	36.23033	21.752476	3.971441	28.10783	44.35284	.000	80.000
	Total	66	32.45121	25.104332	3.090130	26.27980	38.62263	.000	100.000
OR2	PMSG	18	28.48500	18.193542	4.288259	19.43756	37.53244	.000	62.500
	pFSH	10	32.50000	28.360336	8.968326	12.21224	52.78776	.000	100.000
	Total	28	29.91893	21.916924	4.141909	21.42043	38.41742	.000	100.000
OR3	PMSG	6	19.44500	26.177245	10.686815	-8.02633	46.91633	.000	66.670
	pFSH	4	41.25000	13.174587	6.587293	20.28629	62.21371	25.000	57.140
	Total	10	28.16700	23.776854	7.518902	11.15806	45.17594	.000	66.670

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	785.497	1	785.497	1.251	.268
	Within Groups	40179.291	64	627.801		
	Total	40964.788	65			
OR2	Between Groups	103.630	1	103.630	.209	.651
	Within Groups	12865.862	26	494.841		
	Total	12969.492	27			
OR3	Between Groups	1141.099	1	1141.099	2.313	.167
	Within Groups	3946.950	8	493.369		
	Total	5088.049	9			

Appendix Table 2.10: Percentage of Grade B oocyte retrieved from does superstimulated with PMSG vs. pFSH within each OR cycle (Experiment 1)

Descriptives

Grade B

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	PMSG	36	28.16806	30.414307	5.069051	17.87733	38.45878	.000	100.000
	pFSH	30	41.37767	20.713003	3.781660	33.64330	49.11203	.000	100.000
	Total	66	34.17242	27.081993	3.333563	27.51484	40.83001	.000	100.000
OR2	PMSG	18	29.48667	17.201095	4.054337	20.93276	38.04057	.000	60.000
	pFSH	10	20.02400	17.027847	5.384678	7.84301	32.20499	.000	50.000
	Total	28	26.10714	17.443109	3.296438	19.34341	32.87087	.000	60.000
OR3	PMSG	6	16.66500	18.255593	7.452815	-2.49307	35.82307	.000	33.330
	pFSH	4	26.78750	23.600163	11.800081	-10.76563	64.34063	.000	50.000
	Total	10	20.71400	19.953146	6.309739	6.44038	34.98762	.000	50.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	2855.354	1	2855.354	4.077	.048
	Within Groups	44817.879	64	700.279		
	Total	47673.233	65			
OR2	Between Groups	575.628	1	575.628	1.959	.173
	Within Groups	7639.448	26	293.825		
	Total	8215.076	27			
OR3	Between Groups	245.916	1	245.916	.590	.465
	Within Groups	3337.236	8	417.155		
	Total	3583.152	9			

Appendix Table 2.11: Percentage of Grade C oocyte retrieved from does superstimulated with PMSG vs. pFSH within each OR cycle (Experiment 1)

Descriptives

Grade C

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	PMSG	36	32.25472	27.236840	4.539473	23.03910	41.47034	.000	100.000
	pFSH	30	16.44200	19.566429	3.572325	9.13578	23.74822	.000	66.670
	Total	66	25.06712	25.163679	3.097435	18.88112	31.25313	.000	100.000
OR2	PMSG	18	22.24889	20.454003	4.821055	12.07735	32.42043	.000	62.500
	pFSH	10	25.11900	19.331112	6.113034	11.29036	38.94764	.000	50.000
	Total	28	23.27393	19.746925	3.731818	15.61687	30.93099	.000	62.500
OR3	PMSG	6	26.38833	22.617799	9.233678	2.65241	50.12426	.000	50.000
	pFSH	4	15.71500	12.008387	6.004194	-3.39302	34.82302	.000	28.570
	Total	10	22.11900	19.043332	6.022030	8.49622	35.74178	.000	50.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	4091.599	1	4091.599	7.065	.010
	Within Groups	37067.099	64	579.173		
	Total	41158.699	65			
OR2	Between Groups	52.956	1	52.956	.131	.720
	Within Groups	10475.453	26	402.902		
	Total	10528.408	27			
OR3	Between Groups	273.408	1	273.408	.731	.417
	Within Groups	2990.428	8	373.804		
	Total	3263.836	9			

Appendix Table 2.12: Percentage of Grade D oocyte retrieved from does superstimulated with PMSG vs. pFSH within each OR cycle (Experiment 1)

Descriptives

Grade D

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	PMSG	36	8.88472	13.166133	2.194356	4.42994	13.33950	.000	50.000
	pFSH	30	5.18000	8.645736	1.578488	1.95163	8.40837	.000	25.000
	Total	66	7.20076	11.408129	1.404244	4.39629	10.00523	.000	50.000
OR2	PMSG	18	11.53667	14.811455	3.491094	4.17110	18.90223	.000	50.000
	pFSH	10	16.52400	15.639011	4.945490	5.33653	27.71147	.000	50.000
	Total	28	13.31786	15.019192	2.838361	7.49402	19.14169	.000	50.000
OR3	PMSG	6	23.61167	29.068279	11.867075	-6.89362	54.11695	.000	66.670
	pFSH	4	16.25000	19.737865	9.868933	-15.15735	47.65735	.000	40.000
	Total	10	20.66700	24.773732	7.834142	2.94494	38.38906	.000	66.670

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	224.590	1	224.590	1.745	.191
	Within Groups	8234.861	64	128.670		
	Total	8459.451	65			
OR2	Between Groups	159.901	1	159.901	.701	.410
	Within Groups	5930.655	26	228.102		
	Total	6090.556	27			
OR3	Between Groups	130.066	1	130.066	.193	.672
	Within Groups	5393.574	8	674.197		
	Total	5523.640	9			

Appendix Table 2.13: Percentage of Grade E oocyte retrieved from does superstimulated with PMSG vs. pFSH within each OR cycle (Experiment 1)

Descriptives

Grade E

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
OR1	PMSG	36	1.38889	8.333333	1.388889	-1.43071	4.20848	.000	50.000
	pFSH	30	.80300	2.471644	.451258	-.11993	1.72593	.000	9.090
	Total	66	1.12258	6.340753	.780493	-.43618	2.68133	.000	50.000
OR2	PMSG	18	8.24111	17.021252	4.011948	-.22336	16.70558	.000	66.670
	pFSH	10	5.83300	12.452800	3.937921	-3.07520	14.74120	.000	33.330
	Total	28	7.38107	15.345672	2.900059	1.43064	13.33150	.000	66.670
OR3	PMSG	6	13.88833	22.152084	9.043550	-9.35885	37.13552	.000	50.000
	pFSH	4	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	10	8.33300	18.001543	5.692588	-4.54453	21.21053	.000	50.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
OR1	Between Groups	5.617	1	5.617	.138	.712
	Within Groups	2607.717	64	40.746		
	Total	2613.334	65			
OR2	Between Groups	37.279	1	37.279	.153	.699
	Within Groups	6320.941	26	243.113		
	Total	6358.221	27			
OR3	Between Groups	462.926	1	462.926	1.509	.254
	Within Groups	2453.574	8	306.697		
	Total	2916.500	9			

Appendix Table 2.14: Percentage of caprine oocyte retrieved from LOPU vs. Abattoir source for each oocyte grade (Experiment 2)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Grade A	LOPU	14	39.24071	15.361739	4.105598	30.37111	48.11032	11.110	66.670
	Abattoir	6	18.44500	4.879917	1.992218	13.32384	23.56616	11.540	25.470
	Total	20	33.00200	16.227311	3.628537	25.40738	40.59662	11.110	66.670
Grade B	LOPU	14	40.06929	10.604320	2.834124	33.94653	46.19204	22.220	55.560
	Abattoir	6	32.30167	5.051742	2.062365	27.00019	37.60314	26.850	39.740
	Total	20	37.73900	9.848543	2.202201	33.12974	42.34826	22.220	55.560
Grade C	LOPU	14	15.61143	14.173496	3.788026	7.42790	23.79496	.000	55.560
	Abattoir	6	33.79333	6.196288	2.529624	27.29073	40.29594	28.130	44.440
	Total	20	21.06600	14.853596	3.321365	14.11430	28.01770	.000	55.560
Grade D	LOPU	14	4.98357	6.621994	1.769802	1.16015	8.80700	.000	18.180
	Abattoir	6	15.45833	5.083587	2.075366	10.12344	20.79323	8.330	23.150
	Total	20	8.12600	7.813960	1.747255	4.46895	11.78305	.000	23.150
No of oocyte	LOPU	14	11.00000	3.210560	.858058	9.14628	12.85372	5.000	17.000
	Abattoir	6	20.15833	2.918975	1.191667	17.09506	23.22161	16.000	24.000
	Total	20	13.74750	5.275950	1.179738	11.27828	16.21672	5.000	24.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Grade A	Between Groups	1816.339	1	1816.339	10.259	.005
	Within Groups	3186.847	18	177.047		
	Total	5003.187	19			
Grade B	Between Groups	253.411	1	253.411	2.870	.107
	Within Groups	1589.471	18	88.304		
	Total	1842.882	19			
Grade C	Between Groups	1388.443	1	1388.443	8.915	.008
	Within Groups	2803.514	18	155.751		
	Total	4191.957	19			
Grade D	Between Groups	460.827	1	460.827	11.862	.003
	Within Groups	699.275	18	38.849		
	Total	1160.101	19			
No of oocyte	Between Groups	352.275	1	352.275	35.905	.000
	Within Groups	176.602	18	9.811		
	Total	528.877	19			

Appendix Table 2.15: Percentage of caprine oocyte retrieved according to oocyte grades within each oocyte source (Experiment 2)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
LOPU	Grade A	14	39.24071	15.361739	4.105598	30.37111	48.11032	11.110	66.670
	Grade B	14	40.06929	10.604320	2.834124	33.94653	46.19204	22.220	55.560
	Grade C	14	15.61143	14.173496	3.788026	7.42790	23.79496	.000	55.560
	Grade D	14	4.98357	6.621994	1.769802	1.16015	8.80700	.000	18.180
	Total	56	24.97625	19.340395	2.584469	19.79686	30.15564	.000	66.670
Abattoir	Grade A	6	18.44500	4.879917	1.992218	13.32384	23.56616	11.540	25.470
	Grade B	6	32.30167	5.051742	2.062365	27.00019	37.60314	26.850	39.740
	Grade C	6	33.79333	6.196288	2.529624	27.29073	40.29594	28.130	44.440
	Grade D	6	15.45833	5.083587	2.075366	10.12344	20.79323	8.330	23.150
	Total	24	24.99958	9.681321	1.976191	20.91152	29.08765	8.330	44.440

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LOPU	Between Groups	12861.544	3	4287.181	28.910	.000
	Within Groups	7711.255	52	148.293		
	Total	20572.799	55			
Abattoir	Between Groups	1587.891	3	529.297	18.642	.000
	Within Groups	567.853	20	28.393		
	Total	2155.743	23			

Post Hoc Tests

Homogeneous Subsets

LOPU

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
Grade D	14	4.98357		
Grade C	14		15.61143	
Grade A	14			39.24071
Grade B	14			40.06929
Sig.		1.000	1.000	.858

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 14.000.

Abattoir

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
Grade D	6	15.45833	
Grade A	6	18.44500	
Grade B	6		32.30167
Grade C	6		33.79333
Sig.		.343	.633

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix Table 2.16: Percentage of oocyte maturation for LOPU vs. Abattoir oocyte within each IVM duration screened (Experiment 2)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
15 hr	LOPU	3	6.66667	11.547005	6.666667	-22.01768	35.35102	.000	20.000
	Abattoir	3	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	6	3.33333	8.164966	3.333333	-5.23527	11.90194	.000	20.000
18 hr	LOPU	3	53.33333	11.547005	6.666667	24.64898	82.01768	40.000	60.000
	Abattoir	3	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	6	26.66667	30.110906	12.292726	-4.93279	58.26612	.000	60.000
21 hr	LOPU	3	86.66667	11.547005	6.666667	57.98232	115.35102	80.000	100.000
	Abattoir	3	53.33333	30.550505	17.638342	-22.55833	129.22499	20.000	80.000
	Total	6	70.00000	27.568098	11.254629	41.06906	98.93094	20.000	100.000
24 hr	LOPU	3	73.33333	11.547005	6.666667	44.64898	102.01768	60.000	80.000
	Abattoir	3	80.00000	20.000000	11.547005	30.31725	129.68275	60.000	100.000
	Total	6	76.66667	15.055453	6.146363	60.86694	92.46640	60.000	100.000
27 hr	LOPU	3	46.66667	11.547005	6.666667	17.98232	75.35102	40.000	60.000
	Abattoir	3	73.33333	23.094011	13.333333	15.96463	130.70204	60.000	100.000
	Total	6	60.00000	21.908902	8.944272	37.00802	82.99198	40.000	100.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
15 hr	Between Groups	66.667	1	66.667	1.000	.374
	Within Groups	266.667	4	66.667		
	Total	333.333	5			
18 hr	Between Groups	4266.667	1	4266.667	64.000	.001
	Within Groups	266.667	4	66.667		
	Total	4533.333	5			
21 hr	Between Groups	1666.667	1	1666.667	3.125	.152
	Within Groups	2133.333	4	533.333		
	Total	3800.000	5			
24 hr	Between Groups	66.667	1	66.667	.250	.643
	Within Groups	1066.667	4	266.667		
	Total	1133.333	5			
27 hr	Between Groups	1066.667	1	1066.667	3.200	.148
	Within Groups	1333.333	4	333.333		
	Total	2400.000	5			

Appendix Table 2.17: Percentage of oocyte maturation at different IVM durations within each oocyte source (Experiment 2)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
LOPU	15 hr	3	6.66667	11.547005	6.666667	-22.01768	35.35102	.000	20.000
	18 hr	3	53.33333	11.547005	6.666667	24.64898	82.01768	40.000	60.000
	21 hr	3	86.66667	11.547005	6.666667	57.98232	115.35102	80.000	100.000
	24 hr	3	73.33333	11.547005	6.666667	44.64898	102.01768	60.000	80.000
	27 hr	3	46.66667	11.547005	6.666667	17.98232	75.35102	40.000	60.000
	Total	15	53.33333	29.920530	7.725448	36.76390	69.90277	.000	100.000
ABATTOIR	15 hr	3	.00000	.000000	.000000	.00000	.00000	.000	.000
	18 hr	3	.00000	.000000	.000000	.00000	.00000	.000	.000
	21 hr	3	53.33333	30.550505	17.638342	-22.55833	129.22499	20.000	80.000
	24 hr	3	80.00000	20.000000	11.547005	30.31725	129.68275	60.000	100.000
	27 hr	3	73.33333	23.094011	13.333333	15.96463	130.70204	60.000	100.000
	Total	15	41.33333	39.617216	10.229121	19.39405	63.27262	.000	100.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LOPU	Between Groups	11200.000	4	2800.000	21.000	.000
	Within Groups	1333.333	10	133.333		
	Total	12533.333	14			
ABATTOIR	Between Groups	18240.000	4	4560.000	12.214	.001
	Within Groups	3733.333	10	373.333		
	Total	21973.333	14			

Post Hoc Tests

Homogeneous Subsets

LOPU

Duncan[a]

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
15 hr	3	6.66667			
27 hr	3		46.66667		
18 hr	3		53.33333	53.33333	
24 hr	3			73.33333	73.33333
21 hr	3				86.66667
Sig.		1.000	.496	.060	.188

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

ABATTOIR

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
15 hr	3	.00000	
18 hr	3	.00000	
21 hr	3		53.33333
27 hr	3		73.33333
24 hr	3		80.00000
Sig.		1.000	.137

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix Table 2.18: Maturation rate of caprine oocyte from LOPU vs. Abattoir source within each oocyte grade (Experiment 2)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Overall MII rate	LOPU	14	79.59429	9.599481	2.565569	74.05171	85.13686	63.640	100.000
	Abattoir	6	69.70667	8.946701	3.652476	60.31768	79.09565	53.800	81.300
	Total	20	76.62800	10.282281	2.299188	71.81574	81.44026	53.800	100.000
Grade A	LOPU	14	84.40500	12.499331	3.340587	77.18810	91.62190	66.670	100.000
	Abattoir	6	76.43333	10.340342	4.221427	65.58181	87.28486	63.600	90.500
	Total	20	82.01350	12.209887	2.730214	76.29910	87.72790	63.600	100.000
Grade B	LOPU	14	82.05786	18.291341	4.888567	71.49675	92.61896	50.000	100.000
	Abattoir	6	79.63333	7.538877	3.077734	71.72177	87.54490	67.700	89.700
	Total	20	81.33050	15.658045	3.501245	74.00231	88.65869	50.000	100.000
Grade C	LOPU	14	61.07143	43.684999	11.675307	35.84846	86.29440	.000	100.000
	Abattoir	6	68.96667	12.406235	5.064824	55.94712	81.98621	50.000	85.200
	Total	20	63.44000	36.878398	8.246260	46.18038	80.69962	.000	100.000
Grade D	LOPU	14	28.57143	42.581531	11.380393	3.98558	53.15727	.000	100.000
	Abattoir	6	45.20500	18.379155	7.503259	25.91726	64.49274	16.700	66.700
	Total	20	33.56150	37.291479	8.338628	16.10855	51.01445	.000	100.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Overall MII rate	Between Groups	410.613	1	410.613	4.625	.045
	Within Groups	1598.168	18	88.787		
	Total	2008.781	19			
Grade A	Between Groups	266.899	1	266.899	1.873	.188
	Within Groups	2565.646	18	142.536		
	Total	2832.545	19			
Grade B	Between Groups	24.689	1	24.689	.096	.760
	Within Groups	4633.624	18	257.424		
	Total	4658.313	19			
Grade C	Between Groups	261.806	1	261.806	.184	.673
	Within Groups	25578.502	18	1421.028		
	Total	25840.308	19			
Grade D	Between Groups	1162.038	1	1162.038	.828	.375
	Within Groups	25260.395	18	1403.355		
	Total	26422.433	19			

Appendix Table 2.19: Percentage of caprine oocyte maturation according to oocyte grades within each oocyte source (Experiment 2)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
LOPU	Grade A	14	84.40500	12.499331	3.340587	77.18810	91.62190	66.670	100.000
	Grade B	14	82.05786	18.291341	4.888567	71.49675	92.61896	50.000	100.000
	Grade C	14	61.07143	43.684999	11.675307	35.84846	86.29440	.000	100.000
	Grade D	14	28.57143	42.581531	11.380393	3.98558	53.15727	.000	100.000
	Total	56	64.02643	38.811373	5.186388	53.63267	74.42018	.000	100.000
Abattoir	Grade A	6	76.43333	10.340342	4.221427	65.58181	87.28486	63.600	90.500
	Grade B	6	79.63333	7.538877	3.077734	71.72177	87.54490	67.700	89.700
	Grade C	6	68.96667	12.406235	5.064824	55.94712	81.98621	50.000	85.200
	Grade D	6	45.20500	18.379155	7.503259	25.91726	64.49274	16.700	66.700
	Total	24	67.55958	18.219297	3.718998	59.86625	75.25292	16.700	90.500

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
LOPU	Between Groups	28086.907	3	9362.302	8.890	.000
	Within Groups	54760.841	52	1053.093		
	Total	82847.748	55			
Abattoir	Between Groups	4357.357	3	1452.452	8.864	.001
	Within Groups	3277.327	20	163.866		
	Total	7634.684	23			

Post Hoc Tests

Homogeneous Subsets

LOPU

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
Grade D	14	28.57143	
Grade C	14		61.07143
Grade B	14		82.05786
Grade A	14		84.40500
Sig.		1.000	.077

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 14.000.

Abattoir

Duncan[a]

Treatment	N	Subset for alpha = 0.05	
		1	2
Grade D	6	45.20500	
Grade C	6		68.96667
Grade A	6		76.43333
Grade B	6		79.63333
Sig.		1.000	.187

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix Table 2.20: Percentage of fusion and cleavage for bovine intraspSCNT vs. gaur interspSCNT (Experiment 3)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Fused	intraspSCNT (Bovine-bovine)	6	74.31000	9.401636	3.838202	64.44359	84.17641	60.000	86.210
	interspSCNT (Gaur-bovine)	7	70.43000	7.676288	2.901364	63.33062	77.52938	60.000	83.870
	Total	13	72.22077	8.387210	2.326194	67.15243	77.28911	60.000	86.210
Cleaved	intraspSCNT (Bovine-bovine)	6	77.91167	2.283948	.932418	75.51481	80.30852	75.000	81.480
	interspSCNT (Gaur-bovine)	7	73.74571	5.708887	2.157757	68.46587	79.02555	66.670	80.770
	Total	13	75.66846	4.810587	1.334217	72.76145	78.57547	66.670	81.480

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Fused	Between Groups	48.637	1	48.637	.673	.430
	Within Groups	795.506	11	72.319		
	Total	844.143	12			
Cleaved	Between Groups	56.071	1	56.071	2.783	.123
	Within Groups	221.630	11	20.148		
	Total	277.701	12			

Appendix Table 2.21: *In vitro* developmental rate for bovine intraspSCNT vs. gaur interspSCNT (Experiment 3)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Two cell	intraspSCNT (Bovine-bovine)	6	77.9117	2.28395	.93242	75.5148	80.3085	75.00	81.48
	interspSCNT (Gaur-bovine)	7	73.7457	5.70889	2.15776	68.4659	79.0256	66.67	80.77
	Total	13	75.6685	4.81059	1.33422	72.7615	78.5755	66.67	81.48
Four cell	intraspSCNT (Bovine-bovine)	6	69.8467	6.06915	2.47772	63.4775	76.2158	62.50	77.78
	interspSCNT (Gaur-bovine)	7	69.8686	6.61808	2.50140	63.7479	75.9893	58.33	77.27
	Total	13	69.8585	6.10306	1.69268	66.1704	73.5465	58.33	77.78
Eight cell	intraspSCNT (Bovine-bovine)	6	65.3017	7.76832	3.17140	57.1493	73.4540	56.25	77.78
	interspSCNT (Gaur-bovine)	7	62.5243	10.74193	4.06007	52.5897	72.4589	41.67	73.08
	Total	13	63.8062	9.21498	2.55578	58.2376	69.3747	41.67	77.78
morula	intraspSCNT (Bovine-bovine)	6	46.1167	5.91982	2.41676	39.9042	52.3291	37.50	55.56
	interspSCNT (Gaur-bovine)	7	45.8600	3.60209	1.36146	42.5286	49.1914	41.18	50.00
	Total	13	45.9785	4.59424	1.27421	43.2022	48.7547	37.50	55.56
Compact morula	intraspSCNT (Bovine-bovine)	6	37.0983	4.13523	1.68820	32.7587	41.4380	32.00	44.44
	interspSCNT (Gaur-bovine)	7	34.4643	5.66648	2.14173	29.2237	39.7049	26.67	42.31
	Total	13	35.6800	5.00475	1.38807	32.6557	38.7043	26.67	44.44
Blastocyst	intraspSCNT (Bovine-bovine)	6	20.2917	6.34730	2.59127	13.6306	26.9527	11.11	26.32
	interspSCNT (Gaur-bovine)	7	20.9100	10.31163	3.89743	11.3733	30.4467	6.67	33.33
	Total	13	20.6246	8.36986	2.32138	15.5668	25.6825	6.67	33.33
Hatched blastocyst	intraspSCNT (Bovine-bovine)	6	18.6317	5.23533	2.13732	13.1375	24.1258	11.11	26.32
	interspSCNT (Gaur-bovine)	7	19.0186	8.87485	3.35438	10.8107	27.2264	6.67	29.41
	Total	13	18.8400	7.13036	1.97761	14.5312	23.1488	6.67	29.41

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Two cell	Between Groups	56.071	1	56.071	2.783	.123
	Within Groups	221.630	11	20.148		
	Total	277.701	12			
Four cell	Between Groups	.002	1	.002	.000	.995
	Within Groups	446.967	11	40.633		
	Total	446.968	12			
Eight cell	Between Groups	24.922	1	24.922	.276	.610
	Within Groups	994.069	11	90.370		
	Total	1018.991	12			
Morula	Between Groups	.213	1	.213	.009	.925
	Within Groups	253.072	11	23.007		
	Total	253.284	12			
Compact morula	Between Groups	22.416	1	22.416	.886	.367
	Within Groups	278.155	11	25.287		
	Total	300.570	12			
Blastocyst	Between Groups	1.235	1	1.235	.016	.901
	Within Groups	839.419	11	76.311		
	Total	840.654	12			
Hatched blastocyst	Between Groups	.484	1	.484	.009	.927
	Within Groups	609.622	11	55.420		
	Total	610.105	12			

Post Hoc Tests

Homogeneous Subsets

intraspSCNT

Duncan[a]

	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blast.	6	18.63				
Blastocyst	6	20.29				
Compact morula	6		37.1			
Morula	6			46.12		
Eight cell	6				65.30	
Four cell	6				69.85	
Two cell	6					77.91
Sig.		.613	1.000	1.000	.171	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

interspSCNT

Duncan[a]

	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blastocyst	7	19.02				
Blastocyst	7	20.91				
Compact morula	7		34.46			
Morula	7			45.86		
Eight cell	7				62.52	
Four cell	7				69.87	69.87
Two cell	7					73.75
Sig.		.651	1.000	1.000	.084	.356

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.000.

Appendix Table 2.22: Percentage of maturation, enucleation, fusion and *in vitro* development for caprine reconstructed embryos using oocyte matured at 18- 22 hours vs. 23-27 hours (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Maturation rate	18-22	4	75.20750	6.685424	3.342712	64.56950	85.84550	68.420	81.820
	23-27	5	62.56000	2.889991	1.292443	58.97160	66.14840	57.890	65.200
	Total	9	68.18111	8.085155	2.695052	61.96631	74.39591	57.890	81.820
Enucleation rate	18-22	4	80.20250	6.146936	3.073468	70.42135	89.98365	75.000	88.890
	23-27	5	64.24400	10.608425	4.744232	51.07190	77.41610	53.300	81.820
	Total	9	71.33667	11.881976	3.960659	62.20337	80.46996	53.300	88.890
Fusion rate	18-22	4	82.50000	6.123724	3.061862	72.75579	92.24421	75.000	87.500
	23-27	5	84.06000	10.045795	4.492616	71.58650	96.53350	75.000	100.000
	Total	9	83.36667	8.074497	2.691499	77.16006	89.57327	75.000	100.000
Cleavage rate/Two cell	18-22	4	84.22500	11.052111	5.526056	66.63862	101.81138	75.000	100.000
	23-27	5	67.74000	3.755396	1.679464	63.07706	72.40294	62.500	71.400
	Total	9	75.06667	11.328945	3.776315	66.35847	83.77486	62.500	100.000
Four cell	18-22	4	74.70250	8.092875	4.046437	61.82493	87.58007	66.670	85.710
	23-27	5	60.43600	10.376976	4.640725	47.55128	73.32072	44.440	71.430
	Total	9	66.77667	11.616292	3.872097	57.84759	75.70574	44.440	85.710
Eight cell	18-22	4	65.92000	13.392000	6.696000	44.61034	87.22966	57.140	85.710
	23-27	5	46.38800	7.481478	3.345819	37.09852	55.67748	37.500	57.140
	Total	9	55.06889	14.184951	4.728317	44.16537	65.97241	37.500	85.710
Morula	18-22	4	46.57750	8.751466	4.375733	32.65196	60.50304	37.500	57.140
	23-27	5	24.68200	15.973347	7.143498	4.84847	44.51553	.000	42.860
	Total	9	34.41333	17.013663	5.671221	21.33547	47.49119	.000	57.140

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Maturation rate	Between Groups	355.465	1	355.465	14.856	.006
	Within Groups	167.493	7	23.928		
	Total	522.958	8			
Enucleation rate	Between Groups	565.942	1	565.942	7.030	.033
	Within Groups	563.509	7	80.501		
	Total	1129.451	8			
Fusion rate	Between Groups	5.408	1	5.408	.073	.794
	Within Groups	516.172	7	73.739		
	Total	521.580	8			
Cleavage rate/ Two cell	Between Groups	603.900	1	603.900	9.997	.016
	Within Groups	422.860	7	60.409		
	Total	1026.760	8			
Four cell	Between Groups	452.296	1	452.296	5.048	.059
	Within Groups	627.210	7	89.601		
	Total	1079.506	8			
Eight cell	Between Groups	847.776	1	847.776	7.789	.027
	Within Groups	761.927	7	108.847		
	Total	1609.703	8			
Morula	Between Groups	1065.362	1	1065.362	5.964	.045
	Within Groups	1250.356	7	178.622		
	Total	2315.718	8			

Appendix Table 2.23: Percentage of *in vitro* development from 2-cell to morula stage for caprine reconstructed embryos using oocyte matured within each IVM duration range group (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
18-22 hour	2-cell	4	84.22500	11.052111	5.526056	66.63862	101.81138	75.000	100.000
	4-cell	4	74.70250	8.092875	4.046437	61.82493	87.58007	66.670	85.710
	8-cell	4	65.92000	13.392000	6.696000	44.61034	87.22966	57.140	85.710
	morula	4	46.57750	8.751466	4.375733	32.65196	60.50304	37.500	57.140
	Total	16	67.85625	17.158280	4.289570	58.71325	76.99925	37.500	100.000
23-27 hour	2-cell	5	67.74000	3.755396	1.679464	63.07706	72.40294	62.500	71.400
	4-cell	5	60.43600	10.376976	4.640725	47.55128	73.32072	44.440	71.430
	8-cell	5	46.38800	7.481478	3.345819	37.09852	55.67748	37.500	57.140
	morula	5	24.68200	15.973347	7.143498	4.84847	44.51553	.000	42.860
	Total	20	49.81150	19.357082	4.328375	40.75211	58.87089	.000	71.430

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
18-22 hour	Between Groups	3085.366	3	1028.455	9.274	.002
	Within Groups	1330.733	12	110.894		
	Total	4416.098	15			
23-27 hour	Between Groups	5387.616	3	1795.872	16.594	.000
	Within Groups	1731.620	16	108.226		
	Total	7119.236	19			

Post Hoc Tests

Homogeneous Subsets

18-22 hour

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
morula	4	46.57750		
8-cell	4		65.92000	
4-cell	4		74.70250	74.70250
2-cell	4			84.22500
Sig.		1.000	.261	.225

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

23-27 hour

Duncan[a]

Treatment	N	Subset for alpha = 0.05		
		1	2	3
morula	5	24.68200		
8-cell	5		46.38800	
4-cell	5			60.43600
2-cell	5			67.74000
Sig.		1.000	1.000	.283

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix Table 2.24: Percentage of *in vitro* development for caprine reconstructed embryos activated using (7% EtOH, CD-CHX) vs. (CaI, 6-DMAP) within each embryo developmental stage (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
2-cell	7%EtOH, CD-CHX	4	78.89750	2.552794	1.276397	74.83543	82.95957	76.470	81.250
	CaI, 6-DMAP	4	80.68750	5.771617	2.885808	71.50357	89.87143	75.000	87.500
	Total	8	79.79250	4.240841	1.499364	76.24707	83.33793	75.000	87.500
4-cell	7%EtOH, CD-CHX	4	68.81000	1.625628	.812814	66.22326	71.39674	66.670	70.590
	CaI, 6-DMAP	4	70.78000	3.613705	1.806853	65.02979	76.53021	66.670	75.000
	Total	8	69.79500	2.799653	.989827	67.45443	72.13557	66.670	75.000
8-cell	7%EtOH, CD-CHX	4	57.98750	4.692124	2.346062	50.52128	65.45372	53.850	64.710
	CaI, 6-DMAP	4	57.38500	3.085077	1.542538	52.47595	62.29405	53.850	61.110
	Total	8	57.68625	3.690280	1.304711	54.60110	60.77140	53.850	64.710
Morula	7%EtOH, CD-CHX	4	40.00000	2.462627	1.231314	36.08141	43.91859	37.500	42.860
	CaI, 6-DMAP	4	42.77500	5.380945	2.690472	34.21272	51.33728	38.460	50.000
	Total	8	41.38750	4.148297	1.466644	37.91944	44.85556	37.500	50.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
2-cell	Between Groups	6.408	1	6.408	.322	.591
	Within Groups	119.485	6	19.914		
	Total	125.893	7			
4-cell	Between Groups	7.762	1	7.762	.989	.358
	Within Groups	47.105	6	7.851		
	Total	54.866	7			
8-cell	Between Groups	.726	1	.726	.046	.837
	Within Groups	94.601	6	15.767		
	Total	95.327	7			
Morula	Between Groups	15.401	1	15.401	.880	.385
	Within Groups	105.057	6	17.510		
	Total	120.459	7			

Appendix Table 2.25: Percentage of *in vitro* development from 2-cell to morula stage for caprine reconstructed embryos within each activation treatment group (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
EtOH, CD-CHX	2-cell	4	78.89750	2.552794	1.276397	74.83543	82.95957	76.470	81.250
	4-cell	4	68.81000	1.625628	.812814	66.22326	71.39674	66.670	70.590
	8-cell	4	57.98750	4.692124	2.346062	50.52128	65.45372	53.850	64.710
	morula	4	40.00000	2.462627	1.231314	36.08141	43.91859	37.500	42.860
	Total	16	61.42375	15.131455	3.782864	53.36077	69.48673	37.500	81.250
CaI, 6-DMAP	2-cell	4	80.68750	5.771617	2.885808	71.50357	89.87143	75.000	87.500
	4-cell	4	70.78000	3.613705	1.806853	65.02979	76.53021	66.670	75.000
	8-cell	4	57.38500	3.085077	1.542538	52.47595	62.29405	53.850	61.110
	morula	4	42.77500	5.380945	2.690472	34.21272	51.33728	38.460	50.000
	Total	16	62.90688	15.297514	3.824378	54.75541	71.05834	38.460	87.500

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
EtOH, CD-CHX	Between Groups	3322.694	3	1107.565	118.965	.000
	Within Groups	111.720	12	9.310		
	Total	3434.414	15			
CaI, 6-DMAP	Between Groups	3255.681	3	1085.227	51.164	.000
	Within Groups	254.528	12	21.211		
	Total	3510.209	15			

Post Hoc Tests

Homogeneous Subsets

EtOH, CD-CHX

Duncan[a]

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
morula	4	40.000			
8-cell	4		57.988		
4-cell	4			68.810	
2-cell	4				78.898
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

CaI, 6-DMAP

Duncan[a]

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
morula	4	42.775			
8-cell	4		57.385		
4-cell	4			70.780	
2-cell	4				80.688
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Appendix Table 2.26: Percentage of *in vitro* development for caprine reconstructed embryos cultured in mSOFaa vs. KSOMaa within each embryo development stage (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
2-cell	mSOFaa	6	79.27500	11.502544	4.695894	67.20382	91.34618	68.750	100.000
	KSOMaa	4	78.58000	9.435221	4.717611	63.56646	93.59354	70.590	90.480
	Total	10	78.99700	10.164054	3.214156	71.72607	86.26793	68.750	100.000
4-cell	mSOFaa	6	64.91667	10.100021	4.123316	54.31734	75.51599	50.000	75.000
	KSOMaa	4	68.60000	10.167232	5.083616	52.42167	84.77833	58.820	80.950
	Total	10	66.39000	9.733850	3.078114	59.42682	73.35318	50.000	80.950
8-cell	mSOFaa	6	52.72667	5.758513	2.350903	46.68348	58.76985	43.750	58.330
	KSOMaa	4	57.43750	9.255285	4.627642	42.71028	72.16472	47.060	66.670
	Total	10	54.61100	7.272810	2.299864	49.40835	59.81365	43.750	66.670
Morula	mSOFaa	6	31.68500	4.689404	1.914441	26.76377	36.60623	25.000	37.500
	KSOMaa	4	40.42250	7.158349	3.579174	29.03197	51.81303	33.330	47.620
	Total	10	35.18000	7.046700	2.228362	30.13909	40.22091	25.000	47.620
Blastocyst	mSOFaa	6	.00000	.000000	.000000	.00000	.00000	.000	.000
	KSOMaa	4	3.85000	4.687387	2.343694	-3.60868	11.30868	.000	9.520
	Total	10	1.54000	3.358055	1.061910	-.86221	3.94221	.000	9.520

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
2-cell	Between Groups	1.159	1	1.159	.010	.923
	Within Groups	928.613	8	116.077		
	Total	929.772	9			
4-cell	Between Groups	32.561	1	32.561	.318	.588
	Within Groups	820.170	8	102.521		
	Total	852.731	9			
8-cell	Between Groups	53.261	1	53.261	1.008	.345
	Within Groups	422.783	8	52.848		
	Total	476.044	9			
Morula	Between Groups	183.225	1	183.225	5.559	.046
	Within Groups	263.678	8	32.960		
	Total	446.904	9			
Blastocyst	Between Groups	35.574	1	35.574	4.318	.071
	Within Groups	65.915	8	8.239		
	Total	101.489	9			

Appendix Table 2.27: Percentage of *in vitro* development from 2-cell to blastocyst stage for caprine reconstructed embryos within each IVC medium treatment group (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
mSOFaa	2-cell	6	79.27500	11.502544	4.695894	67.20382	91.34618	68.750	100.000
	4-cell	6	64.91667	10.100021	4.123316	54.31734	75.51599	50.000	75.000
	8-cell	6	52.72667	5.758513	2.350903	46.68348	58.76985	43.750	58.330
	Morula	6	31.68500	4.689404	1.914441	26.76377	36.60623	25.000	37.500
	Blastocyst	6	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	30	45.72067	29.025796	5.299361	34.88226	56.55908	.000	100.000
KSOMaa	2-cell	4	78.58000	9.435221	4.717611	63.56646	93.59354	70.590	90.480
	4-cell	4	68.60000	10.167232	5.083616	52.42167	84.77833	58.820	80.950
	8-cell	4	57.43750	9.255285	4.627642	42.71028	72.16472	47.060	66.670
	Morula	4	40.42250	7.158349	3.579174	29.03197	51.81303	33.330	47.620
	Blastocyst	4	3.85000	4.687387	2.343694	-3.60868	11.30868	.000	9.520
	Total	20	49.77800	27.920375	6.243186	36.71086	62.84514	.000	90.480

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
mSOFaa	Between Groups	22985.058	4	5746.265	99.255	.000
	Within Groups	1447.350	25	57.894		
	Total	24432.408	29			
KSOMaa	Between Groups	13757.590	4	3439.397	48.957	.000
	Within Groups	1053.810	15	70.254		
	Total	14811.399	19			

Post Hoc Tests
Homogeneous Subsets

mSOFaa

Duncan[a]

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Blastocyst	6	.000				
Morula	6		31.685			
8-cell	6			52.727		
4-cell	6				64.917	
2-cell	6					79.275
Sig.		1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

KSOMaa

Duncan[a]

Treatment	N	Subset for alpha = 0.05			
		1	2	3	4
Blastocyst	4	3.850			
Morula	4		40.423		
8-cell	4			57.438	
4-cell	4			68.600	68.600
2-cell	4				78.580
Sig.		1.000	1.000	.079	.113

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Appendix Table 2.28: Percentage of *in vitro* development for caprine reconstructed embryos cultured in Treatment A vs. Treatment B within each embryo development stage (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Two cell	Treatment A	7	76.83714	5.688901	2.150202	71.57579	82.09850	71.430	85.710
	Treatment B	7	77.41857	5.080123	1.920106	72.72024	82.11690	73.330	87.500
	Total	14	77.12786	5.190306	1.387168	74.13106	80.12465	71.430	87.500
Fou rcell	Treatment A	7	68.06000	4.454503	1.683644	63.94027	72.17973	61.900	72.730
	Treatment B	7	68.51143	6.824782	2.579525	62.19956	74.82330	60.000	79.170
	Total	14	68.28571	5.541693	1.481080	65.08604	71.48539	60.000	79.170
Eight cell	Treatment A	7	58.20857	5.984152	2.261797	52.67415	63.74299	50.000	66.670
	Treatment B	7	60.46571	4.171142	1.576544	56.60805	64.32338	53.330	66.660
	Total	14	59.33714	5.092094	1.360919	56.39706	62.27723	50.000	66.670
Morula	Treatment A	7	34.94000	6.212123	2.347962	29.19474	40.68526	28.570	44.440
	Treatment B	7	46.67286	4.375807	1.653900	42.62591	50.71980	41.670	54.170
	Total	14	40.80643	7.981896	2.133252	36.19782	45.41504	28.570	54.170
Blastocyst	Treatment A	7	3.79571	3.885743	1.468673	.20200	7.38943	.000	9.520
	Treatment B	7	19.89571	3.972966	1.501640	16.22133	23.57010	13.330	25.000
	Total	14	11.84571	9.167399	2.450091	6.55262	17.13881	.000	25.000
Hatched blastocyst	Treatment A	7	.00000	.000000	.000000	.00000	.00000	.000	.000
	Treatment B	7	15.58000	6.549626	2.475526	9.52261	21.63739	7.690	25.000
	Total	14	7.79000	9.227730	2.466215	2.46207	13.11793	.000	25.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Two cell	Between Groups	1.183	1	1.183	.041	.844
	Within Groups	349.027	12	29.086		
	Total	350.211	13			
Four cell	Between Groups	.713	1	.713	.021	.886
	Within Groups	398.521	12	33.210		
	Total	399.235	13			
Eight cell	Between Groups	17.831	1	17.831	.670	.429
	Within Groups	319.251	12	26.604		
	Total	337.082	13			
Morula	Between Groups	481.810	1	481.810	16.689	.002
	Within Groups	346.429	12	28.869		
	Total	828.239	13			
Blastocyst	Between Groups	907.235	1	907.235	58.752	.000
	Within Groups	185.301	12	15.442		
	Total	1092.536	13			
Hatched blastocyst	Between Groups	849.577	1	849.577	39.610	.000
	Within Groups	257.386	12	21.449		
	Total	1106.963	13			

Appendix Table 2.29: Percentage of *in vitro* development from 2-cell to hatched blastocyst stage for caprine reconstructed embryos within each IVC treatment group (Experiment 4)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Treatment A	2-cell	7	76.83714	5.688901	2.150202	71.57579	82.09850	71.430	85.710
	4-cell	7	68.06000	4.454503	1.683644	63.94027	72.17973	61.900	72.730
	8-cell	7	58.20857	5.984152	2.261797	52.67415	63.74299	50.000	66.670
	Morula	7	34.94000	6.212123	2.347962	29.19474	40.68526	28.570	44.440
	Blastocyst	7	3.79571	3.885743	1.468673	.20200	7.38943	.000	9.520
	Hatched blast.	7	.00000	.000000	.000000	.00000	.00000	.000	.000
	Total	42	40.30690	30.735691	4.742620	30.72899	49.88482	.000	85.710
Treatment B	2-cell	7	77.41857	5.080123	1.920106	72.72024	82.11690	73.330	87.500
	4-cell	7	68.51143	6.824782	2.579525	62.19956	74.82330	60.000	79.170
	8-cell	7	60.46571	4.171142	1.576544	56.60805	64.32338	53.330	66.660
	Morula	7	46.67286	4.375807	1.653900	42.62591	50.71980	41.670	54.170
	Blastocyst	7	19.89571	3.972966	1.501640	16.22133	23.57010	13.330	25.000
	Hatched blast.	7	15.58000	6.549626	2.475526	9.52261	21.63739	7.690	25.000
	Total	42	48.09071	24.191800	3.732876	40.55202	55.62941	7.690	87.500

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Treatment A	Between Groups	37881.757	5	7576.351	320.792	.000
	Within Groups	850.234	36	23.618		
	Total	38731.991	41			
Treatment B	Between Groups	22989.290	5	4597.858	164.588	.000
	Within Groups	1005.681	36	27.936		
	Total	23994.971	41			

Post Hoc Tests
Homogeneous Subsets

Treatment A

Duncan[a]

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blast.	7	.000				
Blastocyst	7	3.796				
Morula	7		34.94			
8-cell	7			58.20		
4-cell	7				68.06	
2-cell	7					76.837
Sig.		.153	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.000.

Treatment B

Duncan[a]

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blast	7	15.58				
Blastocyst	7	19.89				
Morula	7		46.67			
8-cell	7			60.46		
4-cell	7				68.51	
2-cell	7					77.41
Sig.		.135	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 7.000.

Appendix Table 2.30: Percentage of *in vitro* development for caprine intraspSCNT vs. PA embryos within each embryo development stage (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Two cell	intraspSCNT caprine	5	78.09200	6.037986	2.700269	70.59485	85.58915	73.330	87.500
	PA caprine	5	74.09800	6.087895	2.722590	66.53888	81.65712	66.670	80.490
	Total	10	76.09500	6.091516	1.926306	71.73739	80.45261	66.670	87.500
Four cell	intraspSCNT caprine	5	67.97400	7.440923	3.327682	58.73487	77.21313	60.000	79.170
	PA caprine	5	65.33400	8.692398	3.887358	54.54096	76.12704	60.000	80.000
	Total	10	66.65400	7.754027	2.452039	61.10710	72.20090	60.000	80.000
Eight cell	intraspSCNT caprine	5	61.22000	4.855641	2.171509	55.19093	67.24907	53.330	66.660
	PA caprine	5	54.99800	2.888195	1.291640	51.41183	58.58417	53.330	60.000
	Total	10	58.10900	4.993984	1.579236	54.53652	61.68148	53.330	66.660
Morula	intraspSCNT caprine	5	47.59600	4.621870	2.066963	41.85719	53.33481	42.310	54.170
	PA caprine	5	35.33200	4.472882	2.000333	29.77818	40.88582	30.000	40.000
	Total	10	41.46400	7.756629	2.452861	35.91524	47.01276	30.000	54.170
Blastocyst	intraSCNT caprine	5	17.30800	4.081969	1.825512	12.23957	22.37643	12.500	20.830
	PA caprine	5	14.99800	2.888195	1.291640	11.41183	18.58417	13.330	20.000
	Total	10	16.15300	3.548972	1.122283	13.61422	18.69178	12.500	20.830
Hatched blastocyst	intraspSCNT caprine	5	11.26600	2.281881	1.020488	8.43267	14.09933	7.690	13.300
	PA caprine	5	9.00200	6.190450	2.768453	1.31554	16.68846	5.000	20.000
	Total	10	10.13400	4.557397	1.441176	6.87383	13.39417	5.000	20.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Two cell	Between Groups	39.880	1	39.880	1.085	.328
	Within Groups	294.079	8	36.760		
	Total	333.959	9			
Four cell	Between Groups	17.424	1	17.424	.266	.620
	Within Groups	523.700	8	65.463		
	Total	541.124	9			
Eight cell	Between Groups	96.783	1	96.783	6.064	.039
	Within Groups	127.676	8	15.959		
	Total	224.459	9			
Morula	Between Groups	376.014	1	376.014	18.179	.003
	Within Groups	165.473	8	20.684		
	Total	541.488	9			
Blastocyst	Between Groups	13.340	1	13.340	1.067	.332
	Within Groups	100.017	8	12.502		
	Total	113.357	9			
Hatched blastocyst	Between Groups	12.814	1	12.814	.589	.465
	Within Groups	174.115	8	21.764		
	Total	186.929	9			

Appendix Table 2.31: Percentage of *in vitro* development from 2-cell to hatched blastocyst stage for caprine embryos within each approach (intraspSCNT or PA) (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
intraspSCNT caprine	2-cell	5	78.09200	6.037986	2.700269	70.59485	85.58915	73.330	87.500
	4-cell	5	67.97400	7.440923	3.327682	58.73487	77.21313	60.000	79.170
	8-cell	5	61.22000	4.855641	2.171509	55.19093	67.24907	53.330	66.660
	Morula	5	47.59600	4.621870	2.066963	41.85719	53.33481	42.310	54.170
	Blastocyst	5	17.30800	4.081969	1.825512	12.23957	22.37643	12.500	20.830
	Hatched blast	5	11.26600	2.281881	1.020488	8.43267	14.09933	7.690	13.300
	Total	30	47.24267	25.911891	4.730842	37.56701	56.91833	7.690	87.500
PA caprine	2-cell	5	74.09800	6.087895	2.722590	66.53888	81.65712	66.670	80.490
	4-cell	5	65.33400	8.692398	3.887358	54.54096	76.12704	60.000	80.000
	8-cell	5	54.99800	2.888195	1.291640	51.41183	58.58417	53.330	60.000
	Morula	5	35.33200	4.472882	2.000333	29.77818	40.88582	30.000	40.000
	Blastocyst	5	14.99800	2.888195	1.291640	11.41183	18.58417	13.330	20.000
	Hatched blast	5	9.00200	6.190450	2.768453	1.31554	16.68846	5.000	20.000
	Total	30	42.29367	25.446806	4.645930	32.79167	51.79566	5.000	80.490

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
intraspSCNT caprine	Between Groups	18836.825	5	3767.365	142.494	.000
	Within Groups	634.532	24	26.439		
	Total	19471.357	29			
PA caprine	Between Groups	18028.130	5	3605.626	115.299	.000
	Within Groups	750.528	24	31.272		
	Total	18778.657	29			

Post Hoc Tests
Homogeneous Subsets

intraspSCNTcaprine

Duncan[a]

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blast	5	11.27				
Blastocyst	5	17.31				
Morula	5		47.60			
8-cell	5			61.22		
4-cell	5				67.97	
2-cell	5					78.09
Sig.		.075	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

PA caprine

Duncan[a]

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blast	5	9.00				
Blastocyst	5	14.99				
Morula	5		35.33			
8-cell	5			54.99		
4-cell	5				65.33	
2-cell	5					74.10
Sig.		.103	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

Appendix Table 2.32: Percentage of *in vitro* development for caprine interspSCNT vs. PA embryos within each embryo development stage (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Two cell	interspSCNT caprine	6	78.02500	2.444379	.997914	75.45978	80.59022	73.910	80.770
	PA Bovine	6	73.44500	5.998012	2.448678	67.15047	79.73953	66.670	80.000
	Total	12	75.73500	4.978909	1.437287	72.57155	78.89845	66.670	80.770
Four cell	interspSCNT caprine	6	74.27500	4.071215	1.662066	70.00252	78.54748	70.000	80.770
	PA Bovine	6	63.55500	6.821448	2.784844	56.39633	70.71367	53.330	70.000
	Total	12	68.91500	7.747668	2.236559	63.99237	73.83763	53.330	80.770
Eight cell	interspSCNT caprine	6	63.16167	3.235666	1.320955	59.76604	66.55729	60.610	69.230
	PA Bovine	6	56.72167	3.714945	1.516620	52.82307	60.62026	52.000	60.000
	Total	12	59.94167	4.726834	1.364519	56.93838	62.94495	52.000	69.230
Morula	interspSCNT caprine	6	32.74667	4.390962	1.792603	28.13863	37.35470	26.920	39.130
	PA Bovine	6	36.05500	3.349948	1.367610	32.53945	39.57055	32.000	40.000
	Total	12	34.40083	4.104861	1.184971	31.79273	37.00894	26.920	40.000
Blastocyst	interspSCNT caprine	6	8.53500	1.458009	.595230	7.00491	10.06509	6.060	10.000
	PA Bovine	6	12.55500	4.096846	1.672531	8.25562	16.85438	8.000	20.000
	Total	12	10.54500	3.605941	1.040945	8.25389	12.83611	6.060	20.000
Hatched blastocyst	interspSCNT caprine	6	4.81667	1.450388	.592119	3.29458	6.33876	3.030	6.670
	PA Bovine	6	7.88833	4.389398	1.791964	3.28194	12.49472	.000	13.330
	Total	12	6.35250	3.505290	1.011890	4.12535	8.57965	.000	13.330

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Two cell	Between Groups	62.929	1	62.929	3.000	.114
	Within Groups	209.756	10	20.976		
	Total	272.685	11			
Four cell	Between Groups	344.755	1	344.755	10.926	.008
	Within Groups	315.535	10	31.553		
	Total	660.290	11			
Eight cell	Between Groups	124.421	1	124.421	10.253	.009
	Within Groups	121.352	10	12.135		
	Total	245.773	11			
Morula	Between Groups	32.835	1	32.835	2.153	.173
	Within Groups	152.513	10	15.251		
	Total	185.349	11			
Blastocyst	Between Groups	48.481	1	48.481	5.128	.057
	Within Groups	94.550	10	9.455		
	Total	143.031	11			
Hatched blastocyst	Between Groups	28.305	1	28.305	2.649	.135
	Within Groups	106.852	10	10.685		
	Total	135.158	11			

Appendix Table 2.33: Percentage of *in vitro* development from 2-cell to hatched blastocyst stage for caprine embryos within each approach (interspSCNT or PA) (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
interSCNT caprine	2-cell	6	78.02500	2.444379	.997914	75.45978	80.59022	73.910	80.770
	4-cell	6	74.27500	4.071215	1.662066	70.00252	78.54748	70.000	80.770
	8-cell	6	63.16167	3.235666	1.320955	59.76604	66.55729	60.610	69.230
	Morula	6	32.74667	4.390962	1.792603	28.13863	37.35470	26.920	39.130
	Blastocyst	6	8.70167	1.141252	.465914	7.50400	9.89934	7.060	10.000
	Hatched blast	6	4.81667	1.450388	.592119	3.29458	6.33876	3.030	6.670
	Total	36	43.62111	30.411322	5.068554	33.33140	53.91082	3.030	80.770
PA bovine	2-cell	6	73.44500	5.998012	2.448678	67.15047	79.73953	66.670	80.000
	4-cell	6	63.55500	6.821448	2.784844	56.39633	70.71367	53.330	70.000
	8-cell	6	56.72167	3.714945	1.516620	52.82307	60.62026	52.000	60.000
	Morula	6	36.05500	3.349948	1.367610	32.53945	39.57055	32.000	40.000
	Blastocyst	6	12.55500	4.096846	1.672531	8.25562	16.85438	8.000	20.000
	Hatched blast	6	7.88833	4.389398	1.791964	3.28194	12.49472	.000	13.330
	Total	36	41.70333	25.707856	4.284643	33.00505	50.40162	.000	80.000

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
interSCNT caprine	Between Groups	32091.167	5	6418.233	691.298	.000
	Within Groups	278.530	30	9.284		
	Total	32369.697	35			
PA bovine	Between Groups	22413.374	5	4482.675	187.322	.000
	Within Groups	717.911	30	23.930		
	Total	23131.285	35			

Post Hoc Tests

Homogeneous Subsets

interSCNT caprine

Duncan[a]

Treatment	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
Hatched blast	6	4.82					
Blastocyst	6		8.70				
Morula	6			32.74			
8-cell	6				63.16		
4-cell	6					74.27	
2-cell	6						78.02
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

PA bovine

Duncan[a]

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blast	6	7.89				
Blastocyst	6	12.50				
Morula	6		36.05			
8-cell	6			56.72		
4-cell	6				63.55	
2-cell	6					73.44
Sig.		.109	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix Table 2.34: Percentage of maturation, enucleation, fusion for caprine intraspSCNT vs. interspSCNT approach (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Maturation rate	intraspSCNT caprine	5	79.69000	6.426908	2.874201	71.70994	87.67006	73.080	86.670
	interspSCNT caprine	6	78.04500	4.708438	1.922212	73.10380	82.98620	73.100	86.050
	Total	11	78.79273	5.323980	1.605240	75.21603	82.36943	73.080	86.670
Positive enucleation rate	intraspSCNT caprine	5	90.85800	5.516223	2.466930	84.00870	97.70730	86.360	100.000
	interspSCNT caprine	6	88.05500	5.212672	2.128064	82.58464	93.52536	78.380	93.330
	Total	11	89.32909	5.282067	1.592603	85.78055	92.87763	78.380	100.000
Fusion rate	intraspSCNT caprine	5	82.62400	7.660312	3.425796	73.11247	92.13553	72.730	92.310
	interspSCNT caprine	6	75.26500	2.296717	.937631	72.85474	77.67526	71.880	78.570
	Total	11	78.61000	6.393678	1.927766	74.31467	82.90533	71.880	92.310

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Maturation rate	Between Groups	7.380	1	7.380	.241	.636
	Within Groups	276.068	9	30.674		
	Total	283.448	10			
Positive enucleation rate	Between Groups	21.428	1	21.428	.749	.409
	Within Groups	257.575	9	28.619		
	Total	279.002	10			
Fusion rate	Between Groups	147.695	1	147.695	5.091	.050
	Within Groups	261.096	9	29.011		
	Total	408.791	10			

Appendix Table 2.35: Percentage of *in vitro* development for caprine intraspSCNT vs. interspSCNT approach within each embryo development stage (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Two cell	intraspSCNT caprine	5	78.09200	6.037986	2.700269	70.59485	85.58915	73.330	87.500
	interspSCNT caprine	6	78.02500	2.444379	.997914	75.45978	80.59022	73.910	80.770
	Total	11	78.05545	4.191852	1.263891	75.23933	80.87158	73.330	87.500
Four cell	intraspSCNT caprine	5	67.97400	7.440923	3.327682	58.73487	77.21313	60.000	79.170
	interspSCNT caprine	6	74.27500	4.071215	1.662066	70.00252	78.54748	70.000	80.770
	Total	11	71.41091	6.423574	1.936781	67.09549	75.72633	60.000	80.770
Eight cell	intraspSCNT caprine	5	61.22000	4.855641	2.171509	55.19093	67.24907	53.330	66.660
	interspSCNT caprine	6	63.16167	3.235666	1.320955	59.76604	66.55729	60.610	69.230
	Total	11	62.27909	3.961549	1.194452	59.61769	64.94050	53.330	69.230
Morula	intraspSCNT caprine	5	47.59600	4.621870	2.066963	41.85719	53.33481	42.310	54.170
	interspSCNT caprine	6	32.74667	4.390962	1.792603	28.13863	37.35470	26.920	39.130
	Total	11	39.49636	8.849974	2.668368	33.55087	45.44186	26.920	54.170
Blastocyst	intraspSCNT caprine	5	17.30800	4.081969	1.825512	12.23957	22.37643	12.500	20.830
	interspSCNT caprine	6	8.70167	1.141252	.465914	7.50400	9.89934	7.060	10.000
	Total	11	12.61364	5.245650	1.581623	9.08956	16.13771	7.060	20.830
Hatched blastocyst	intraspSCNT caprine	5	11.26600	2.281881	1.020488	8.43267	14.09933	7.690	13.300
	interspSCNT caprine	6	4.81667	1.450388	.592119	3.29458	6.33876	3.030	6.670
	Total	11	7.74818	3.805049	1.147265	5.19192	10.30445	3.030	13.300

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Two cell	Between Groups	.012	1	.012	.001	.981
	Within Groups	175.704	9	19.523		
	Total	175.716	10			
Four cell	Between Groups	108.280	1	108.280	3.202	.107
	Within Groups	304.343	9	33.816		
	Total	412.623	10			
Eight cell	Between Groups	10.282	1	10.282	.631	.447
	Within Groups	146.657	9	16.295		
	Total	156.939	10			
Morula	Between Groups	601.371	1	601.371	29.763	.000
	Within Groups	181.849	9	20.205		
	Total	783.220	10			
Blastocyst	Between Groups	202.006	1	202.006	24.850	.001
	Within Groups	73.162	9	8.129		
	Total	275.168	10			
Hatched blastocyst	Between Groups	113.438	1	113.438	32.570	.000
	Within Groups	31.346	9	3.483		
	Total	144.784	10			

Appendix Table 2.36: Percentage of *in vitro* development from 2-cell to hatched blastocyst for caprine embryos within each nuclear transfer approach (Experiment 5)

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
intraspSCNT caprine	2-cell	5	78.09200	6.037986	2.700269	70.59485	85.58915	73.330	87.500
	4-cell	5	67.97400	7.440923	3.327682	58.73487	77.21313	60.000	79.170
	8-cell	5	61.22000	4.855641	2.171509	55.19093	67.24907	53.330	66.660
	Morula	5	47.59600	4.621870	2.066963	41.85719	53.33481	42.310	54.170
	Blastocyst	5	17.30800	4.081969	1.825512	12.23957	22.37643	12.500	20.830
	Hatched blast	5	11.26600	2.281881	1.020488	8.43267	14.09933	7.690	13.300
	Total	30	47.24267	25.911891	4.730842	37.56701	56.91833	7.690	87.500
interspSCNT caprine	2-cell	6	78.02500	2.444379	.997914	75.45978	80.59022	73.910	80.770
	4-cell	6	74.27500	4.071215	1.662066	70.00252	78.54748	70.000	80.770
	8-cell	6	63.16167	3.235666	1.320955	59.76604	66.55729	60.610	69.230
	Morula	6	32.74667	4.390962	1.792603	28.13863	37.35470	26.920	39.130
	Blastocyst	6	8.70167	1.141252	.465914	7.50400	9.89934	7.060	10.000
	Hatched blast	6	4.81667	1.450388	.592119	3.29458	6.33876	3.030	6.670
	Total	36	43.62111	30.411322	5.068554	33.33140	53.91082	3.030	80.770

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
intraspSCNT caprine	Between Groups	18836.825	5	3767.365	142.494	.000
	Within Groups	634.532	24	26.439		
	Total	19471.357	29			
interspSCNT caprine	Between Groups	32091.167	5	6418.233	691.298	.000
	Within Groups	278.530	30	9.284		
	Total	32369.697	35			

Post Hoc Tests

Homogeneous Subsets

intraspSCNT caprine

Duncan[a]

Treatment	N	Subset for alpha = 0.05				
		1	2	3	4	5
Hatched blast	5	11.27				
Blastocyst	5	17.31				
Morula	5		47.59			
8-cell	5			61.22		
4-cell	5				67.97	
2-cell	5					78.09
Sig.		.075	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 5.000.

interspSCNT caprine

Duncan[a]

Treatment	N	Subset for alpha = 0.05					
		1	2	3	4	5	6
Hatched blast	6	4.82					
Blastocyst	6		8.701				
Morula	6			32.74			
8-cell	6				63.16		
4-cell	6					74.27	
2-cell	6						78.02
Sig.		1.000	1.000	1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix Table 2.37: Number of cell count in the hatched blastocyst derived from intraspSCNT, interspSCNT, caprine PA and bovine PA (Experiment 5)

Descriptives

Cell no of H. Blast

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Caprine SCNT	5	109.0000	10.55936	4.72229	95.8888	122.1112	97.00	121.00
Caprine iSCNT	5	81.4000	5.77062	2.58070	74.2348	88.5652	73.00	88.00
Caprine PA	4	107.5000	10.08299	5.04149	91.4557	123.5443	97.00	121.00
Bovine PA	5	112.2000	10.35374	4.63033	99.3441	125.0559	98.00	124.00
Total	19	102.2632	15.49137	3.55396	94.7966	109.7298	73.00	124.00

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3006.684	3	1002.228	11.450	.000
Within Groups	1313.000	15	87.533		
Total	4319.684	18			

Post Hoc Tests

Homogeneous Subsets

VAR00001

Duncan[a,,b]

TREATMENT	N	Subset for alpha = 0.05	
		1	2
Caprine interspSCNT	5	81.4000	
Caprine PA	4		107.5000
Caprine intraspSCNT	5		109.0000
Bovine PA	5		112.2000
Sig.		1.000	.477

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.706.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

APPENDIX 3: LIST OF PUBLICATIONS AND PRESENTATIONS

Appendix 3.1: ISI Article Publication

Appendix 3.1.1: **Kwong P.J.**, R.B. Abdullah and W.E. Wan Khadijah. 2012. Increasing glucose in KSOMaa basal medium on culture Day 2 improves *in vitro* development of cloned caprine blastocysts produced via intraspecies and interspecies somatic cell nuclear transfer. Theriogenology. 78: 921-929

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Increasing glucose in KSOMaa basal medium on culture Day 2 improves *in vitro* development of cloned caprine blastocysts produced via intraspecies and interspecies somatic cell nuclear transfer

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Abstract

This study was conducted to evaluate the efficiency of potassium simplex optimization medium with amino acids (KSOMaa) as a basal culture medium for caprine intraspecies somatic cell nuclear transfer (SCNT) and caprine-bovine interspecies somatic cell nuclear transfer (iSCNT) embryos. The effect of increased glucose as an energy substrate for late stage development of cloned caprine embryos *in vitro* was also evaluated. Enucleated caprine and bovine *in vitro* matured oocytes at metaphase II were reconstructed with caprine ear skin fibroblast cells for the SCNT and iSCNT studies. The cloned caprine and parthenogenetic embryos were cultured in either KSOMaa with 0.2 mM glucose for 8 days (Treatment 1) or KSOMaa for 2 days followed by KSOMaa with additional glucose at a final concentration of 2.78 mM for the last 6 days (Treatment 2). There were no significant differences in the cleavage rates of SCNT (80.7%) and iSCNT (78.0%) embryos cultured in KSOMaa medium. Both Treatment 1 and Treatment 2 could support *in vitro* development of SCNT and iSCNT embryos to the blastocyst stage. However, the blastocyst development rate of SCNT embryos was significantly higher ($P < 0.05$) in Treatment 2 compared to Treatment 1. Increasing glucose for later stage embryo development (8-cell stage onwards) during *in vitro* culture (IVC) in Treatment 2 also improved both caprine SCNT and iSCNT embryo development to the hatched blastocyst stage. In conclusion, this study shows that cloned caprine embryos derived from SCNT and iSCNT could develop to the blastocyst stage in KSOMaa medium supplemented with additional glucose (2.78 mM, final concentration) and this medium also supported hatching of caprine cloned blastocysts.

Keywords: Caprine cloned blastocyst; Nuclear transfer; Cross-species SCNT; Embryo culture; KSOMaa; Glucose

1. Introduction

In studies on caprine somatic cell nuclear transfer (SCNT) embryos, most of the reports on successful production of cloned kids at present involved transfer to recipient females of early stage (1- to 2-cell) cloned embryos, so the embryo *in vitro* culture (IVC) duration was as short as 2 days [1–3]. One of the reasons for transferring caprine embryos at these early stages is the difficulty of obtaining viable late stage caprine embryos, particularly blastocysts, after prolonged IVC. However, transfer of blastocysts into the uterus is a less

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invasive embryo transfer method compared to oviductal embryo transfer, which requires surgery. Therefore, the availability of an IVC system that supports caprine reconstructed embryos up to the blastocyst stage is vital and can be expected to improve the production rate of cloned kids. The importance of producing caprine SCNT-derived blastocysts is further highlighted when considering that embryonic stem (ES) cells can be generated with much higher efficiency from blastocysts formed by SCNT [4]. Yet another rationale for developing cloned caprine embryos by IVC is that this approach can serve as a source of material for investigating nuclear reprogramMing and gene regulation mechanisms pertaining to epigenetic memory.

Current reports on producing cloned caprine blastocysts *in vitro* mainly used mSOF [5–7], CR1aa [8] and TCM 199 [9] as basal culture media. There has been no reported attempt to apply KSOMaa as the IVC medium for caprine embryo culture. The KSOM was originally formulated by Lawitts and Biggers [10] for mouse embryo culture. An improvement on this medium, mKSOMaa, was formulated by SumMers, et al. [11,12] with the addition of glucose (5.56 mM), BSA (4 mg/mL) and amino acids [13] to increase the development rate of mouse IVF embryos. Since mKSOMaa supports mouse embryo development *in vitro* favorably, this culture medium was then tested in other domestic animals, such as cattle [14] and pigs [15] using a low glucose concentration (0.2 mM). In this medium, both bovine and porcine embryos could develop up to the blastocyst stage.

Addition of glucose or increasing its concentration in the culture medium for late stages of embryo development (morula, blastocyst) is reported to improve blastocyst production in murine, ovine and bovine IVC studies [16–18]. However, while glucose in the embryo culture medium enhances blastocyst production *in vitro*, the optimal concentration varies for different species and from one laboratory to another. Furnus, et al. [19] reported that increasing the glucose concentration above 1.5 mM in mSOF increased the bovine blastocyst development rate, but at 5 mM concentration glucose was detrimental. This finding is in contrast to the report by Kim, et al. [20] who reported that glucose at 2.78 to 5.56 mM was favorable for increasing bovine blastocyst rates. In a study on mouse embryo culture, SumMers, et al. [11] reported that glucose at 5.56 mM did not significantly inhibit blastocyst development. Thus, in view of widely varying reports on the optimal glucose concentration in different species, for the present study we selected a glucose concentration of 2.78 mM which is intermediate among the three reports mentioned above, and compared it to the basal glucose concentration of 0.2 mM in medium KSOMaa.

The aim of this study was to evaluate the potential of KSOMaa medium for *in vitro* development (IVD) of caprine SCNT and interspecies somatic cell nuclear transfer (iSCNT) embryos and the effect of additional glucose in the culture medium.

2. Materials and methods

All chemicals were obtained from Sigma-Aldrich, Co (St. Louis, MO, USA) unless otherwise specifically indicated. EmCare holding medium (ICP Bio, Auckland, New Zealand) was used as the manipulation medium for all manipulations under a normal air atmosphere.

2.1. Oocyte retrieval

For the caprine SCNT study, caprine oocytes were obtained from abattoir-derived ovaries and also via the laparoscopic ovum pick up (LOPU) technique on superstimulated does, while for caprine iSCNT the source of bovine oocytes was entirely from abattoir-derived ovaries. The abattoir-derived caprine and bovine ovaries were transported to the laboratory in 0.9% NaCl supplemented with penicillin-G (60 μg/mL) and streptomycin (50 μg/mL) at 30 to 37 °C. All the ovaries were rinsed at least four times with 0.9% NaCl. The surfaces of the ovaries were sliced with a razor blade in a checkered pattern to retrieve cumulus-oocyte-complexes (COCs). The COCs were collected using sterile modified Dulbecco's phosphate buffered saline (mDPBS). LOPU was performed using the estrus synchronization regime of a 14 days progesterone vaginal implant (CIDR-G, Pharmacia & Upjohn, Ltd., New Zealand) (300 mg) followed by injection of cloprostenol (Estrumate, Schering-Plough, Australia) (125 μg) i.m. at 36 h before CIDR removal. To superstimulate the does, FSH (Folltropin-V, Vetpharm, Canada) (70 mg) and LH (Ovidrel, Serono, Switzerland) (250 IU) were administered i.m. at 65 and 60 h, respectively, before LOPU. Then, LOPU was carried out according to the procedure described by Abdullah, et al. [21].

2.2. In vitro maturation of caprine and bovine oocytes

The collected COCs were washed 4 times in mDPBS droplets followed by washing 5 times in *in vitro* maturation (IVM) medium before culturing them in 50 μL droplets of IVM medium (TCM 199 supple-

mented with 5 μg/mL FSH, 1 μg/mL 17-β estradiol, 22 μg mL^{-1} sodium pyruvate, 85 μg mL^{-1} cysteine, 50 μg mL^{-1} gentamicin and 10% fetal bovine serum (FBS)) overlaid with mineral oil under a humidified atmosphere of 5% CO_2 in air at 38.5 °C. Caprine oocytes derived from LOPU were cultured in IVM medium for 18 to 22 h while caprine and bovine oocytes retrieved from abattoir-derived ovaries were cultured for 22 to 24 h.

2.3. Donor cell preparation for nuclear transfer

The donor cell preparation method was adapted from Suteevun, et al. [22]. Caprine ear skin fibroblast (EF) cells were utilized as the donor cells for the production of caprine SCNT and iSCNT embryos. The caprine ear skin tissues were biopsied and washed in mDPBS. Then, after removing cartilage from the skin tissues, they were chopped into small pieces (approximately 1 mM^3) before culture in a 60 mM culture dish. The explants were cultured for 8 to 10 days in α-MEM supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 in air at 37 °C. Then, the EF cells were harvested using 0.25% trypsin/EDTA and subcultured until passage 3. The EF cells at passage 3 were harvested and cryopreserved using 10% dimethyl sulfoxide (DMSO) mixed into the tissue culture medium, and stored in liquid nitrogen. The frozen cells were thawed and cultured up to 80% confluence, 2 days before cloning.

2.4. Nuclear transfer

The nuclear transfer protocol in this study was adapted from Abdullah, et al. [23]. Mature bovine and caprine oocytes with a first polar body were treated with 5 μg/mL cytochalasin B for 15 min before enucleation. A squeezing technique was incorporated into this enucleation step. A cut was made in the zona pellucida above the polar body and using mild pressure from a glass needle, 5 to 10% of the cytoplasm beneath the polar body was squeezed out together with the polar body. The extruded polar body and cytoplasm were stained with 5 μg/mL Hoechst 33342 and viewed under a fluorescence microscope to confirm enucleation. Subsequently, a single EF cell (14–16 μM in diameter) was injected into the perivitelline space of an enucleated oocyte. The donor cell-recipient cytoplast couplet was sandwiched between a pair of electrodes in ZimMermann fusion medium [24], and two direct current pulses (21 V, 15 μs) were applied generated by an SUT F-1 fusion machine manufactured by Suranaree University of Technology, Thailand, for cell-cytoplast fusion. This was an optimized fusion protocol obtained by the authors for caprine SCNT in the laboratory (unpublished data). Both caprine SCNT and iSCNT approaches used the same fusion parameters. Successfully fused couplets were subjected to chemical activation in 5 μM calcium ionophore for 5 min followed by 2 mM 6-dimethylaminopurine (DMAP) for 4 h, then incubated at 38.5 °C under a humidified atmosphere of 5% CO_2 in air.

2.5. Parthenogenetic activation (PA)

In each replicate, some of the mature caprine and bovine oocytes obtained were directly activated with 5 μM calcium ionophore for 5 min followed by 2 mM DMAP for 4 h and incubated at 38.5 °C under a humidified atmosphere of 5% CO_2 in air before transfer into IVC medium.

2.6. In vitro embryo culture

The caprine reconstructed embryos (SCNT and iSCNT) and PA embryos (as controls) were cultured in two different IVC medium treatments designated as follows:

Treatment 1—IVC in KSOMaa with 0.2 mM glucose for 8 days (8D).

Treatment 2—IVC in KSOMaa with 0.2 mM glucose for 2 days (2D) + KSOMaa with 2.78 mM glucose (final concentration) for another 6 days (6D).

The experimental design adapted the formula of KSOMaa developed by Ho, et al. [13] and SumMers, et al. [11,12] with modification of the glucose content as shown above, and a two-step culture system was used in IVC Treatment 2.

The glucose concentration of 2.78 mM was selected for this study based on the report by Kim, et al. [20] who showed that bovine blastocyst development rate was increased using glucose at 2.78 to 5.56 mM in mTLP-PVA medium together with 0.35 mM phosphate and amino acids, which are contained in KSOMaa. In this study, both IVC medium treatments were supplemented with 4 mg/mL BSA and all the embryos were incubated under a humidified atmosphere of 5% CO_2 in air at 38.5 °C. Half the volume of the IVC medium in the culture drop was replaced with fresh medium every 2 days starting from Day 2 onwards for both treatments. The first observation of embryo cleavage was made on Day 2 post-activation. The efficiency of these two IVC medium treatments was evaluated by comparing the cleavage and blastocyst rates of caprine reconstructed embryos and of caprine and bovine PA embryos.

2.7. Fluorescent staining for enumeration of embryonic cell number

Caprine SCNT, iSCNT and PA-derived hatched blastocysts were subjected to fluorescent staining according to methods described by Uhm, et al. [25]. In brief, the hatched blastocysts were fixed for 5 min in fixative solution consisting of 2% (v/v) formaldehyde and 0.25% (v/v) glutaraldehyde. The fixed, hatched blastocysts were then mounted on clean glass slides and stained with glycerol-based Hoechst 33 342 (12.5 μg/mL) solution for 10 min. Stained nuclei appeared blue when visualized under UV illumination of an epifluorescent microscope fitted with a standard blue filter.

2.8. Experimental design

In this study, there were 3 sub-experiments. In Experiment 1, the fusion rates for both SCNT and iSCNT were examined using a standardized fusion protocol of two direct current pulses (21 V, 15 μs) and the cleavage rates after fusion for both approaches were evaluated. In Experiment 2 the effects of two IVC medium treatments (Treatment 1 vs. Treatment 2) on the development of caprine SCNT and iSCNT embryos were evaluated. The development rates reported in this study were obtained by calculating the percentage of embryos developing divided by the number of fused couplets. In Experiment 3, the cell numbers of cloned caprine SCNT, iSCNT and PA hatched blastocysts were enumerated by counting the number of stained nuclei observed under UV illumination. All the stained hatched blastocysts were derived from embryos cultured in Treatment 2 because none of the embryos in Treatment 1 developed to this stage.

Oocytes collected from abattoir ovaries and via LOPU were randomly allotted to all treatment groups mentioned above. On the same days as the SCNT and iSCNT experiments, caprine or bovine PA embryos, respectively, were cultured as controls.

2.9. Statistical analyses

Data were analyzed using SPSS Version 12. The differences among mean percentages for fusion, cleavage and *in vitro* developmental rates for caprine SCNT iSCNT and PA embryos using two IVC media treatments were analyzed by one-way ANOVA followed by Duncan's Multiple Range Test. Significance was determined at $P < 0.05$.

3. Results

3.1. Fusion and cleavage rates of caprine SCNT and iSCNT embryos (Experiment 1)

The fusion rate with the caprine SCNT approach was significantly higher than with iSCNT ($P < 0.05$), but, there was no significant difference in cleavage rates between the two approaches (Table 1).

3.2. In vitro development of reconstructed SCNT and iSCNT embryos cultured in two different IVC media (Experiment 2)

The development rates of caprine SCNT embryos cultured in IVC Treatment 1 vs. Treatment 2 are shown in Table 2. Development in the two groups was similar ($P > 0.05$) at the early stages (2-cell to 8-cells). However, development to later stages (morula to hatched blastocyst) was greater in Treatment 2 ($P < 0.05$). None of the caprine SCNT embryos developed into hatched blastocyst in Treatment 1. The development of caprine SCNT embryos cultured in either Treatment 1 or 2 did not differ significantly from their respective control group (caprine PA embryos) at any stage from 2-cell to hatched blastocyst.

Table 3 depicts the development rates of caprine iSCNT embryos cultured in IVC medium Treatment 1 vs. Treatment 2. The iSCNT embryos cultured in Treatment 2 had significantly higher ($P < 0.05$) development at the 8-cell, blastocyst and hatched blastocyst stages compared to those cultured in Treatment 1. Similar to the caprine SCNT embryos cultured in Treatment 1, none of the iSCNT blastocysts hatched in Treatment 1. Development of all stages of caprine iSCNT embryos cultured in either treatment was similar to the respective control group (bovine PA embryos), except for development to 4- and 8-cells in Treatment 2, which was significantly

Table 1
Fusion and cleavage rates (mean ± SEM) for caprine SCNT and iSCNT reconstructed embryos.

Type of NT approach	Number of replicates	Fusion rate* (n)	Cleavage rate (n)
Caprine SCNT(caprine-caprine)	10	83.2 ± 2.8^{b} (159/190)	80.7 ± 4.3^{a} (128/159)
Caprine iSCNT (caprine-bovine)	6	75.3 ± 0.9^{a} (165/219)	78.0 ± 1.0^{a} (129/165)

* Based on numbers of oocytes subjected to electrofusion.
[a,b] Means with different superscripts in a column are significantly different ($P < 0.05$).

Table 2
In vitro development (mean ± SEM) of caprine SCNT embryos cultured in two different IVC media.

IVC medium treatment	Type of caprine embryo	2-cells	4-cells	8-cells	Morula	Blastocyst	Hatched blastocyst
Treatment 1	SCNT	75.0 ± 2.6[a] (55/74)	65.4 ± 3.1[a] (48/74)	54.8 ± 3.5[a] (40/74)	31.6 ± 1.8[a] (23/74)	3.9 ± 2.3[a] (3/74)	0.0 ± 0.0[a] (0/74)
	PA	80.8 ± 6.7[a] (31/40)	67.5 ± 4.8[a] (26/40)	54.2 ± 3.4[a] (21/40)	30.0 ± 4.1[a] (11/40)	1.7 ± 1.7[a] (1/40)	0.0 ± 0.0[a] (0/40)
Treatment 2	SCNT	80.6 ± 4.3[a] (128/159)	73.8 ± 5.0[a] (116/159)	66.1 ± 4.4[a] (101/159)	44.3 ± 3.2[b] (70/159)	20.7 ± 4.0[b] (26/159)	15.0 ± 4.3[b] (18/159)
	PA	72.6 ± 3.0[a] (46/65)	61.7 ± 1.7[a] (40/65)	53.8 ± 0.4[a] (35/65)	34.2 ± 2.1[ab] (22/65)	13.8 ± 0.4[ab] (9/65)	6.3 ± 0.4[ab] (4/65)

Treatment 1: KSOMaa (8 days); Treatment 2: KSOMaa (2 days) +KSOMaa with 2.78 mM glucose (6 days).
[a,b] Means with different superscripts in a column are significantly different ($P < 0.05$).

higher. The photomicrographs of the SCNT and iSCNT caprine embryos are depicted in Fig. 1.

3.3. Cell numbers of caprine SCNT, iSCNT and PA hatched blastocysts (Experiment 3)

Caprine SCNT, iSCNT and PA hatched blastocyst cell numbers were assessed (Table 4). The mean cell number of caprine hatched blastocysts derived from iSCNT (81.4) was significantly lower ($P < 0.05$) compared to SCNT (109), caprine PA (107.3) and bovine PA (112.2) hatched blastocysts. There were no significant differences among cell numbers of the latter 3 groups. Photomicrographs of stained caprine SCNT and iSCNT hatched blastocysts are depicted in Fig. 2 a, b, respectively.

4. Discussion

The fusion rate with the iSCNT approach was significantly lower than that with SCNT using the same fusion parameters. Perhaps optimization of fusion parameters that suit the caprine-bovine iSCNT approach is required because cytoplasts and karyoplasts are derived from different species. However, the cleavage rate for fused embryos from both SCNT approaches did not differ significantly, thus indicating that the bovine cytoplast has the potential to promote dedifferentiation of caprine nuclei (karyoplasts) with an efficiency similar to that of the caprine cytoplast. The ability of bovine cytoplast to dedifferentiate karyoplasts of various mamMalian species was also demonstrated in the study by Dominko, et al. [26].

The results obtained in this study demonstrated that reconstructed caprine embryos, regardless of whether they are derived from SCNT or iSCNT, could develop up to the hatched blastocyst stage using KSOMaa provided that the glucose concentration was increased. The developmental outcome was improved in our laboratory when using KSOMaa compared to our previous study [23] using mSOF as the IVC medium in which both SCNT and iSCNT embryos only developed up to the morula stage.

Table 3
In vitro development (mean ± SEM) of caprine iSCNT embryos cultured in two different IVC media.

IVC medium treatment	Type of embryo	2-cells	4-cells	8-cells	Morula	Blastocyst	Hatched blastocyst
Treatment 1	Caprine iSCNT	75.6 ± 1.6[a] (54/71)	67.2 ± 2.3[ab] (48/71)	56.0 ± 2.3[a] (40/71)	35.9 ± 3.6[a] (26/71)	2.5 ± 1.5[a] (2/71)	0.0 ± 0.0[a] (0/71)
	Bovine PA	72.5 ± 4.8[a] (33/45)	63.3 ± 3.3[a] (29/45)	55.0 ± 2.9[a] (25/45)	35.0 ± 2.9[a] (16/45)	3.3 ± 3.3[ab] (2/45)	0.0 ± 0.0[a] (0/45)
Treatment 2	Caprine iSCNT	78.0 ± 1.0[a] (129/165)	74.3 ± 1.7[b] (122/165)	63.2 ± 1.3[b] (104/165)	32.8 ± 1.8[a] (54/165)	8.5 ± 0.6[bc] (14/165)	4.8 ± 0.6[b] (8/165)
	Bovine PA	73.5 ± 2.5[a] (84/115)	63.6 ± 2.8[a] (73/115)	56.7 ± 1.5[a] (65/115)	36.1 ± 1.4[a] (41/115)	12.6 ± 1.7[c] (14/115)	7.9 ± 1.8[b] (10/115)

Treatment 1: KSOMaa (8 days); Treatment 2: KSOMaa (2 days) +KSOMaa with 2.78 mM glucose (6 days).
[a,b,c] Means with different superscripts in a column are significantly different ($P < 0.05$).

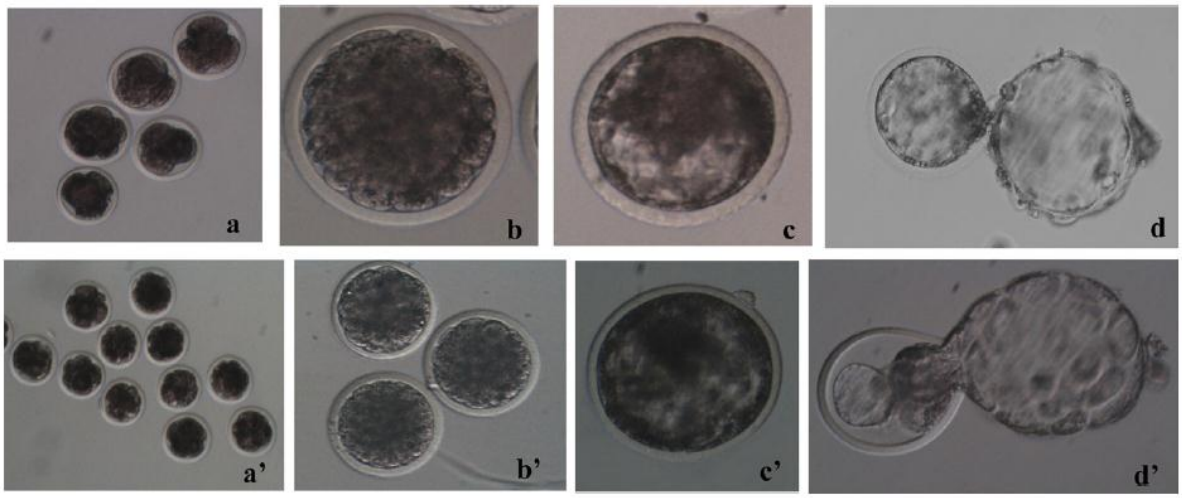

Fig. 1. Caprine SCNT (a−d) and iSCNT (a'−d') embryos at: 4- to 8-cell (a, a'), morula (b, b'), blastocyst (c, c') and hatched blastocyst (d, d') stages.

However, when effects of Treatments 1 and 2 on the development rate of caprine SCNT and iSCNT embryos were analyzed, significant effects of the IVC medium treatments were observed on the later embryo stages (morula and blastocyst). Before this, by Day 2 post-activation, most of the caprine SCNT, iSCNT and PA embryos cultured in either IVC medium treatments developed to the 8-cell stage with no significant differences among them, as expected because for the first 2 days of IVC, Treatments 1 and 2 shared the same formulation of KSOMaa.

The cleavage rate of caprine cloned embryos cultured in KSOMaa in this study is significantly improved compared to reports by other researchers using mSOF [5,23,27] and CR1aa [8]. Possibly, the improvement was due to the presence of EDTA in KSOMaa: a similar beneficial effect of EDTA on early stages of embryo development was reported in murine [28–30] and bovine [31,32] embryos.

Table 4
Cell number (mean ± SEM) of cloned caprine and PA hatched blastocysts cultured in Treatment 2.

Type of embryo	Number of hatched blastocysts stained	Cell Number ± SEM
Caprine SCNT	5	109 ± 4.7[a]
Caprine iSCNT	5	81.4 ± 2.6[b]
Caprine PA	4	107.3 ± 5.1[a]
Bovine PA	5	112.2 ± 4.6[a]

[a,b] Means with different superscripts in a column are significantly different ($P < 0.05$).

The requirement for glucose in the culture medium for early stages of embryo development is controversial. Some reports stated that the presence of glucose during the first day of IVC is unfavorable to embryos in several species, namely mouse [33], sheep [17] and cattle [34]. However, KSOMaa basal medium used on Day 1 and Day 2 of embryo culture in the present study contained 0.2 mM glucose and yet a high cleavage rate of cloned caprine embryos was obtained. This agrees with the findings by Matsuyama, et al. [35] on bovine embryo *in vitro* development. At this stage of development, even though glucose is not being used as the main energy substrate, however, a low level of glycolysis does occur [36]; moreover, glucose is required in the biosynthesis of nucleic acids [37], which are essential for embryonic development at this early stage.

The IVD of cloned caprine embryos was observed to reach 8-cell and morula stages at 48 and 96 h post-activation. Similar timeline was also reported in the IVD of monkey iSCNT and bovine SCNT embryos [38]. Telford, et al. [39] and Rieger, et al. [18], studying changes in the metabolism of glucose by bovine embryos throughout *in vitro* development, demonstrated that glucose metabolism does increased from 8-cell stage onwards. A similar observation on glucose intake was also reported in sheep embryos [40]. Thus, in this study, changing of IVC medium at Day 2 with increased glucose concentration might be beneficial to the IVD of the 8-cell stage

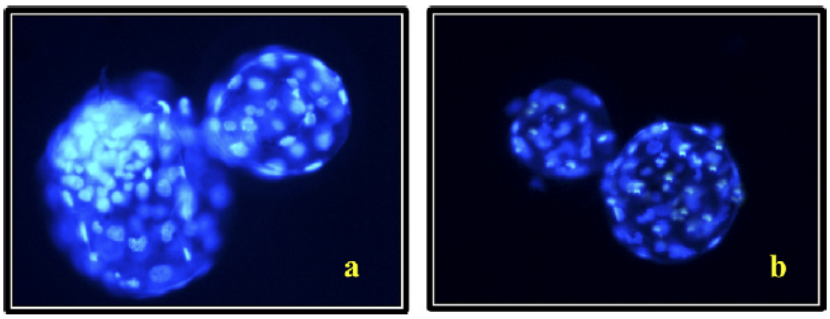

Fig. 2. Cloned caprine hatched blastocyst stained with Hoechst 33342: (a) SCNT; (b) iSCNT.

embryos to the subsequent stages. Furthermore, glucose is also beneficial for blastocyst expansion because energy derived from glycolysis is required for maintaining the blastocoel [18,41]. This explains why, in Treatment 1 without additional glucose, the percentage of SCNT blastocysts was significantly lower than in Treatment 2 and none of the cloned or PA blastocysts hatched.

Comparing the cloning efficiency between SCNT and iSCNT approaches cultured in both Treatments 1 and 2, the embryo development rates obtained in this study at morula stage onwards indicate that the iSCNT approach is still not as efficient as SCNT. Even though the culture system developed in this study could support the development of caprine iSCNT blastocysts, their viability, i.e., ability to develop to term, is still unknown. Further studies related to the compatibility of mitochondrial and genomic DNA, embryonic genome activation of the donor nucleus (karyoplast) by the recipient oocyte (cytoplast) and the availability of suitable foster mothers for embryo transfer to carry the iSCNT embryos to term need to be carried out.

At present, all the available reports on successful production of cloned caprine offspring used intraspecies SCNT [1–3]. None of the domestic animals has been successfully cloned using intergeneric SCNT. Most of the cattle [42], buffalo [43], sheep [26] and goat [44,45] intergeneric SCNT embryos produced only managed to develop up to the blastocyst stage. The blastocyst development rate for caprine iSCNT (8.5%) using bovine cytoplasts in the present study is similar to the report by Song, et al. [45] using caprine fetal fibroblast cells and bovine ooplasm (7.9%), while in comparison with caprine iSCNT (2.2%) using buffalo cytoplasts [44], the blastocyst rate in this study is slightly higher. As for the production of cloned caprine blastocysts using SCNT, Tang, et al. [5] reported that by using fetal fibroblast cells with mSOF-FBS as the IVC medium, the blastocyst rate could reach 24.3% which is slightly higher than in our study (20.7%).

In this study, the cell number of caprine iSCNT blastocysts was lower than in caprine SCNT and PA blastocysts. Similar observations were reported in other iSCNT studies, such as in bovine-buffalo [46] iSCNT. It is important to examine the blastocyst cell number because the developmental competence of embryos to term after embryo transfer correlates with the number of cells present in the blastocyst. Fleming, et al. [47] demonstrated that low blastocyst cell numbers are implicated in reduced development and may lead to large offspring syndrome.

5. Conclusions

KSOMaa basal medium can be used as an alternative to currently available IVC media for caprine SCNT blastocyst production, and the increased glucose concentration (2.78 mM) introduced into KSOMaa medium at Day 2 of IVC significantly increases blastocyst and hatched blastocyst formation rates.

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Comparison of intra- and interspecies nuclear transfer techniques in the production of cloned caprine embryos

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ABSTRACT

Among the ARTs that are applied in goat farming industry, reproductive cloning technology in production of cloned goat embryos are foreseen to facilitate the effort of mass goat production in just a short time frame. There are two possible approaches that can be applied to produce cloned embryos, namely intraspecies SCNT (intraSCNT) and interspecies SCNT (interSCNT). The application of interSCNT is known to play vital role in species preservation, livestock propagation and therapeutic cloning. In fact, the application of interSCNT approach to produce cloned caprine embryos has not been reported at present. The prospect of this application can be seen to overcome the relative difficulty of obtaining caprine oocytes to be used as recipient cytoplasm in the cloning protocol. Several studies have shown that ooplasm of bovine can support early development of embryos produced by nuclear transfer using somatic cell nuclei derived from different mammalian species such as sheep, pigs and rats. Therefore, this present study was conducted with the aim to produce cloned caprine embryos using intraSCNT versus interSCNT technique. As a control to this experiment, bovine intraSCNT was conducted. The source of bovine and caprine oocytes was obtained from the abattoir-derived ovaries and also via laparoscopy ovum pick-up (LOPU) technique on superovulated does. The collected oocytes were subsequently cultured in *in vitro* maturation medium for 18–22 h. The matured oocytes were then subjected to enucleation process. The enucleated oocytes were then injected with either a male ear fibroblast cell from caprine or bovine. The couplets were electrofused and chemically activated before *in vitro* cultured. The results for fusion rate of caprine interSCNT (64.2%) was significantly lower compared to the caprine intraSCNT (81.9%). The reconstructed caprine oocytes derived from interSCNT approach seemed to have the developmental efficiency that is comparable to the intraSCNT approach as the cleavage rate of both caprine intra- (48.9%) and interSCNT (51.3%) embryos did not differ significantly. The *in vitro* development of caprine interSCNT could not go beyond morula stage. Therefore the comparison of the *in vitro* developmental rate of cloned bovine and caprine embryos using the intra- and inter-SCNT approach was made up to morula stage. The percentage of cloned caprine embryos developed to morula using intra- (20.6%) and interSCNT (6.9%) approach did not differ significantly. However, the percentage of cloned bovine morula derived from intraSCNT approach (46.1%) was significantly higher. Generally, caprine embryos are known to have a lower *in vitro* developmental potential towards the late perimplantation stage. Even the reports of success in producing cloned kids involved the transfer of embryos at early cloned embryos stages from 2 to 8 cell stages. In the nutshell, cloned caprine embryos can be produced via both intraSCNT and interSCNT approach. The efficacy of interSCNT approach is comparable to the intraSCNT approach in an effort to produce early preimplantation stages of cloned caprine embryos.

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1. Introduction

The application of assisted reproduction technologies (ARTs) in goat breeding program becomes a limelight in the 21st century. The yearly increasing demand of goat meat consumption and its dairy products worldwide, served as the moving gear in goat breeding industries to multiply the goat population via the application of ARTs besides sustaining the conventional breeding program. Among the ARTs that are applied in goat farming industry, reproductive cloning technology in production of cloned goat embryos are foreseen to facilitate the effort of mass goat production in just a short time frame. Reproductive cloning using somatic cell nuclear transfer (SCNT) approach has been applied in the effort of cloning various animals like sheep (Wilmut et al., 1997), cattle (Cibelli et al., 1998), mouse (Wakayama et al., 1998), goat (Baguisi et al., 1999) and pig (Polejaeva et al., 2000).

Up to date, the success rate of SCNT applied on caprine species is still low compared to other domestic animals like the bovine and ovine. Therefore there are still many areas of research that can be carried out to increase the success rate of caprine SCNT. At present, there are two possible approaches that can be applied to produce cloned embryos, namely intraspecies SCNT (intraSCNT) and interspecies SCNT (interSCNT). The application of interSCNT is known to play vital role in species preservation, livestock propagation and therapeutic cloning. In fact, the application of interSCNT approach to produce cloned caprine embryos has not been reported at present. The prospect of this application can be seen to overcome the relative difficulty of obtaining caprine oocytes to be used as recipient cytoplasm in the cloning protocol. Several studies have shown that ooplasm of bovine can support early development of embryos produced by nuclear transfer using somatic cell nuclei derived from different mammalian species such as sheep, pigs, rats (Dominko et al., 1999) and gaur (Lanza et al., 2000). Therefore, this present study was conducted with the aim to produce cloned caprine embryos using intraSCNT versus interSCNT technique.

2. Materials and methods

All chemicals were obtained from Sigma–Aldrich Co. (St. Louis, Mo, USA) unless otherwise specifically indicated. Each experiment consisted of at least six replicates and for each replicate, oocytes were from the same group of abattoir-derived bovine ovaries and laparoscopy ovum pick-up (LOPU)-derived goat oocytes collected on the same day.

2.1. Oocyte retrieval and in vitro maturation

For the oocyte preparation, the source of bovine oocytes was obtained from the abattoir-derived ovaries while caprine oocytes were obtained via LOPU technique on superovulated does. The abattoir-derived bovine ovaries were transported back to the laboratory in sterile modified Dulbecco's phosphate buffered saline (mDPBS) at 30–37 °C supplemented with penicillin-G (60 μg/ml) and Streptomycin (50 μg/ml). Cumulus-oocyte-complexes (COCs) were collected from follicles 2–3 mm in diameter by aspiration using a 21 gauge needle attached to a 10 ml syringe. The collected COCs were washed 4 times in mDPBS droplets followed by 5 times washing in *in vitro* maturation (IVM) medium before cultured in 50 μl droplets of IVM medium (TCM 199 supplemented with 10 μg/ml FSH, 10 μg/ml LH, 1 μg/ml 17-β estradiol, 100 μM/ml cysteamine, 10% fetal bovine serum) overlaid with mineral oil under a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 22 h.

The source of caprine oocytes were collected via LOPU on synchronized and superovulated does using the regime of 14 days progesterone vaginal implant (CIDR-G® 300 mg, Pharmacia & Upjohn Limited, New Zealand) followed by injection of cloprostenol (Estrumate®, 125 μg, Schering-Plough, Australia) at 36 h prior CIDR removal. In order to superovulate the doe, pregnant mare serum gonadotrophin (PMSG, 1200 IU) and ovidrel (250 IU) were administered at 66 h and 71 h, respectively, prior to LOPU. The collected caprine COCs were cultured in the same IVM medium used for bovine oocytes maturation for 18–22 h under a humidified atmosphere of 5% CO_2 in air at 38.5 °C.

2.2. Donor cell preparation for nuclear transfer

For the donor cell preparation, the method used was adapted from Suteevun et al. (2006). The ear fibroblast (EF) cells derived from bovine and caprine were utilized as the donor cell for the production of intraSCNT and interSCNT cloned caprine and bovine embryos. The bovine and caprine ear skin tissues were biopsied from a bull and a buck. The tissues were washed in mDPBS. Then the cartilage were removed from the skin tissues and chopped into small pieces (approximately 1 mm^2) prior cultured in a 60 mm culture dish. The explants were cultured for 8–10 days in αMEM supplemented with 10% fetal

Table 1
Percentage (mean ± SEM) of success in enucleation, injection, fusion and cleavage rate of intraSCNT and interSCNT bovine and caprine cloned embryos.

Type of SCNT approach	No. of replicate	Percentage of oocyte successfully enucleated (*n*)	Percentage of oocyte successfully injected with donor karyoplast (*n*)	Percentage of couplets successfully fused[a] (*n*)	Percentage of reconstructed embryos cleaved[a] (*n*)
intraSCNT (bovine–bovine)	6	89.2 ± 8.2 (162/181)	95.9 ± 6.7 (157/162)	74.3 ± 9.4^{xy} (119/157)	77.7 ± 2.3^{x} (93/119)
intraSCNT (caprine–caprine)	11	95.9 ± 3.1 (57/60)	97.6 ± 1.6 (55/57)	81.9 ± 5.6^{x} (43/55)	48.9 ± 7.8^{y} (21/43)
interSCNT (caprine–bovine)	13	94.4 ± 2.2 (215/228)	97.7 ± 1.2 (207/215)	64.2 ± 3.2^{y} (144/207)	51.3 ± 5.6^{y} (74/144)

^{x,y}Means with different superscripts in a column were significantly different ($P \leq 0.05$).
[a] Only fusion and cleavage rate were analyzed with one-way ANOVA.

Table 2
Percentage (mean ± SEM) of *in vitro* developmental rate for intraSCNT and interSCNT bovine and caprine cloned embryos.

Type of SCNT approach	Percentage of cleaved reconstructed SCNT embryos according to cell stage			
	2-Cell	4-Cell	8-Cell	Morula
intraSCNT (bovine–bovine)	77.7 ± 2.3^{a,x} (93/119)	69.9 ± 2.5^{b,x} (83/119)	65.3 ± 3.2^{b,x} (77/119)	46.1 ± 2.4^{c,x} (54/119)
intraSCNT (caprine–caprine)	48.9 ± 7.8^{a,y} (21/43)	42.6 ± 6.8^{a,y} (18/43)	34.4 ± 7.0^{ab,y} (15/43)	20.6 ± 6.7^{b,y} (9/43)
interSCNT (caprine–bovine)	50.3 ± 3.6^{a,y} (74/144)	46.3 ± 3.2^{a,y} (63/144)	36.6 ± 3.3^{b,y} (49/144)	6.9 ± 2.1^{c,y} (11/144)

^{a,b,c,d}Means with different superscripts in a row were significantly different ($P \leq 0.05$).
^{x,y}Means with different superscripts in a column were significantly different ($P \leq 0.05$).

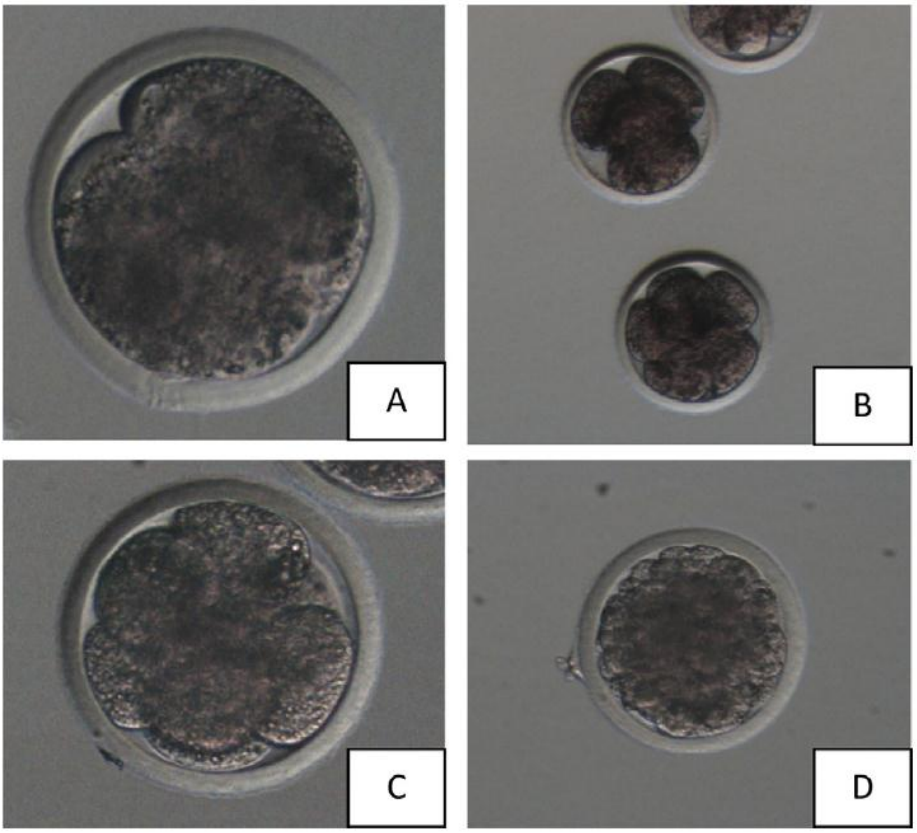

Fig. 1. Caprine intraSCNT embryos at 2-cell stage (A), 4–8-cell stages (B and C), and morula (D).

bovine serum (FBS) under a humidified atmosphere of 5% CO_2 in air at 37 °C. The EF cells were harvested using 0.25% trypsin/EDTA and then subcultured until passage 3. The EF cells at passage 3 were harvested and cryopreserved using 10% dimethyl sulfoxide (DMSO) mixed in the tissue culture medium and stored in liquid nitrogen. The frozen cells were thawed and cultured up to 80% confluence, 2 days prior to cloning.

2.3. Nuclear transfer

For the somatic cell nuclear transfer, bovine and caprine COCs after IVM treatment were denuded in 0.2% hyaluronidase and subsequently washed 5 times in a commercial embryo holding medium (EmCare®). Only matured oocytes with the first polar body were subjected to a 15 min treatment of 5 μg/ml cytochalasin B prior to enucleation. The matured oocytes were enucleated using the squeezing technique. A cut was made on the zona pellucide above the first polar body and 10% of the cytoplasm beneath the first polar body was squeezed out using a glass needle. The squeezed out cytoplasm was subjected to 5 μg/ml Hoechst 33345 stain and viewed under the fluorescence microscope in order to confirm a positive enucleation. Then, a single EF cell was injected into the perivitelline space of an enucleated oocyte. In the approach of caprine or bovine intraSCNT, a caprine or bovine EF cell were injected into an enucleated caprine or bovine oocyte, respectively, while for caprine interSCNT, it involved the transfer of a caprine EF cell into an enucleated bovine oocyte. The donor cell-recipient cytoplast couplets were then fused in Zimmermann fusion medium (Zimmermann and Vienken, 1982) with the parameter of 22–24 V, 2 direct current (DC) pulse and 15 μsec using the fusion machine SUT F-1 manufactured by Suranaree University of Technology, Thailand.

2.4. Activation

For the activation of reconstructed oocytes, the fused couplets were activated in 5 μM calcium ionophore for 5 min and followed with 1.9 μM 6-dimethylaminopurine (DMAP) for 5 h.

2.5. In vitro culture

For the *in vitro* culture of reconstructed oocyte, the reconstructed oocytes were cultured in modified synthetic oviductal fluid (mSOF) under a humidified atmosphere of 5% CO_2 in air at 38.5 °C. The first observation of embryo cleavage was made on Day-2 of post-activation. Replacement of fresh medium on the embryo culture droplets was made every 2 days starting from Day-2 onwards.

2.6. Statistical analyses

Data were analyzed using SPSS version 12. The differences among mean percentages for fusion rate, cleavage rate and *in vitro* developmental rate for both intraSCNT and interSCNT protocols were analyzed by one-way ANOVA followed by Duncan Multiple Range Test. Significance was determined at $P \leq 0.05$.

3. Results and discussion

The fusion rate and cleavage rate for cloned caprine embryos using intraSCNT and interSCNT approach are shown in Table 1. As a control for this experiment, fusion rate and cleavage rate of bovine intraSCNT were compared. The results obtained showed that the fusion rate

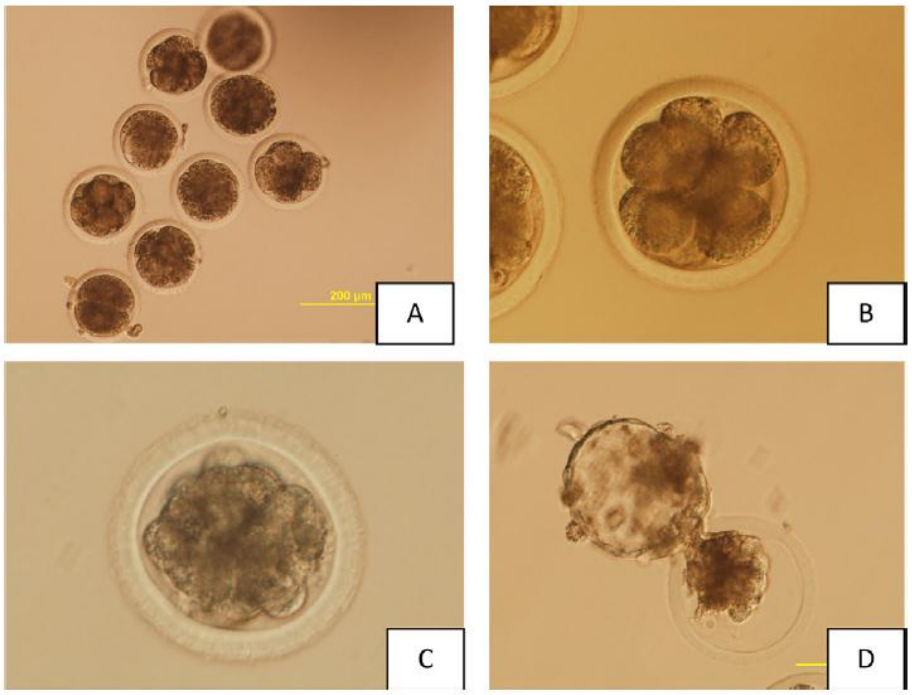

Fig. 2. Bovine intraSCNT embryos at Day-2 post-activation (A), bovine intraSCNT embryo at 8-cell (B), compact morula (C) and hatched blastocyst (D) stages (*Note*: this is only achieved in the bovine intraSCNT).

of caprine interSCNT (64.2%) was significantly lower compared to the caprine intraSCNT (81.9%). The lower fusion efficiency in caprine interSCNT might be due to the species difference factor as the fusion rate of intraSCNT approach for both caprine and bovine species did not differ significantly. However, the fusion rate obtained in this present study for the caprine intraSCNT seems to be higher compared with the rate reported previously by Daniel et al. (2008). The reconstructed caprine oocytes derived from interSCNT approach seemed to have the developmental efficiency that is comparable to the intraSCNT approach as the cleavage rate of both caprine intra- (48.9%) and interSCNT (51.3%) embryos did not differ significantly. This has shown that bovine ooplasm have the potential to dedifferentiate caprine somatic cell nuclei.

In Table 2, the *in vitro* development of the cleaved cloned caprine embryos using intra- and interSCNT approach were evaluated. The *in vitro* development of caprine interSCNT could not go beyond morula stage. This finding is similar to the monkey iSCNT as reported by Lorthongpanich et al. (2008). Therefore the comparison of the *in vitro* developmental rate of cloned bovine and caprine embryos using the intra- and interSCNT approach in this study was made up to morula stage. The *in vitro* development of intraSCNT approach for caprine and bovine is shown in Figs. 1 and 2. While for the caprine interSCNT derived embryos are shown in Fig. 3. The percentage of cloned caprine embryos developed to morula using intra- (20.6%) and interSCNT (6.9%) approach did not differ significantly. However, the percentage of cloned bovine morula derived from

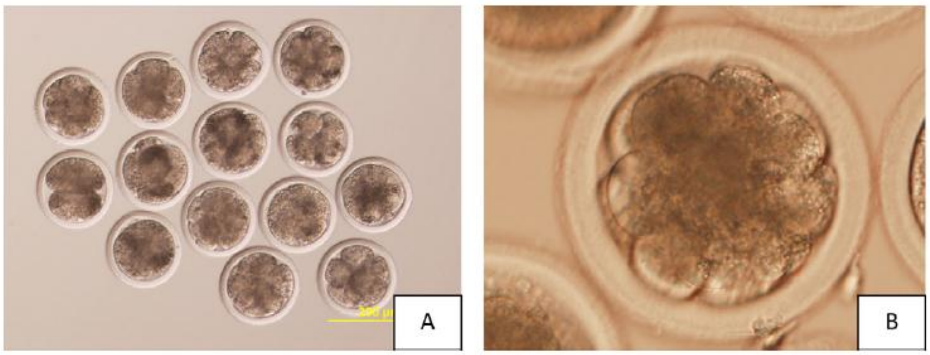

Fig. 3. Caprine interSCNT embryos at Day-2 post-activation (A), caprine interSCNT cloned embryo at morula stage (B).

intraSCNT approach (46.1%) was significantly higher compared to the percentage of cloned caprine morula obtained. Generally, caprine embryos are known to have a lower *in vitro* developmental potential towards the late stage of preimplantation development (Das et al., 2003). Even the reports of success in producing cloned kids involved the transfer of embryos at early cloned caprine embryos stages from 2 to 8 cell stages (Melican et al., 2005; Keefer et al., 2002). Since the *in vitro* developmental efficiency of caprine intraSCNT and interSCNT embryos at all cell stages does not differ significantly, there might be a potential for the caprine interSCNT embryos to develop beyond morula if embryo transfer could be performed at the early preimplantation stage as many researchers successfully obtained caprine intraSCNT offspring by transferring early stage cloned caprine embryos into surrogate does.

4. Conclusions

From the results of this study, cloned caprine embryos can be produced *in vitro* via both intraSCNT and interSCNT approaches in which the efficacy of interSCNT approach is comparable to that of intraSCNT approach.

Conflict of interest

None declared.

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Appendix 3.2: Proceeding (Oral Presentation)

Appendix 3.2.1: **Kwong, P.J**., W.E Wan Khadijah and R.B. Abdullah. 2011. Production of cloned caprine blastocyst using intra- and interspecies SCNT approach. Proceeding of the 32nd Annual Conference of Malaysian Society of Animal Production (MSAP), June 6-9, Promenade Hotel, Tawau, Sabah, Malaysia. pp. 89-90 (Abstract).

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PRODUCTION OF CLONED CAPRINE BLASTOCYSTS USING INTRA- AND INTERSPECIES SCNT APPROACH

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The emerging of successful reports on producing various cloned animal such as bovine, ovine,caprine and porcine since 1997 has led to the interest of many researchers in applying nuclear transfer technology not only in basic research but also in biomedical, agriculture and wildlife conservation fields. Generally, somatic cell nuclear transfer (SCNT) can be carried out using two approaches namely the intraspecies SCNT (intraSCNT) and interspecies SCNT (interSCNT). The available reports up-to-date on the production of cloned caprine embryos and offspring is through the integration of intraSCNT approach (1), and there are no any significant report by other researcher available on the production of cloned caprine via interSCNT approach, specifically using bovine oocytes as recipient cytoplastexcept the attempt conducted by our laboratory (2).This interSCNT approach is indeed useful to enable production of cloned embryos in circumstances where scarce oocyte resources of the domestic goats arise. Today, interSCNT using bovine oocyte as recipient cytoplasm is not being integrated solely to mass propagate superior animal for commercialisation purposes. Indeed its importance and potentials are foreseen to facilitate the conservation effort of endangered animals like gaur and banteng (3, 4). Therefore, in this present study, bovine oocyte is being integrated as the recipient cytoplasm to produce cloned caprine embryos with the futuristic view that this interSCNTapproach can be applied not only to save extinct caprine species like the serow goat but also as a technology to revitalise the present caprine breeding industry. Furthermore, most of the reports on successful production of cloned kids available were accomplished by transferring 2- and 4-cell cloned caprine embryos into the surrogate mothers (5), and not many reported on the *in vitro* developmental (IVD) competency of cloned caprine embryos up to blastocyst stage.

Therefore, the main aim of this study was to elucidate the possibility of producing cloned caprine blastocyst in *in vitro* culture (IVC) system via somatic cell nuclear transfer (SCNT) technique in Malaysia, while the sub-objectives comprise of: (i) to evaluate the IVD competency of LOPU-derived caprine oocytes versus abattoir-derived caprine oocytes and (ii) to compare the efficacy of intraSCNT (caprine-caprine) versus interSCNT (caprine-bovine) approach in producing cloned caprine blastocyst. The source of bovine oocytes used in interSCNT (caprine-bovine) was obtained from abattoir. The manipulation technique of SCNT used in this study involvedenucleation via squeezing technique followed by injection of caprine ear fibroblast cell into the perivitelline space of the enucleated oocytes and finally fused with electrofusion machine before subjected to chemical activation treatment and IVC. The data obtained were analysed using SPSS programme, means were analysed using ANOVA and differences among means were determine using DMRT. Significance was determined at $P \leq 0.05$.

In this present study, the *in vitro* maturation (IVM) rate for caprine oocytes derived from LOPU was significantly higher (77.5±3.37) compared to abattoir-derived caprine oocytes (63.11±1.69), while the IVM rate of bovine oocytes did not differ

significantly (73.9±1.41) compared to the overall caprine IVM rate (71.74±2.80) using the same IVC system. The cleavage rate, blastocyst rate and hatched blastocyst rate (77.55±5.14, 17.99±5.54 and 11.96±4.13) obtained for cloned caprineintraSCNT using LOPU-derived oocytes, respectively, did not differ significantly compared to using abattoir-derived oocytes (76.6±2.13, 9.52±1.75 and 5.69±1.78). A similar scenario was observed when comparison on cleavage rate, blastocyst rate and hatched blastocyst rate were made between intraSCNT approach (77.17±3.12, 14.60±3.49 and 9.45±2.64) and interSCNT approach (79.16±1.64, 6.29±1.24 and 3.5±0.89), where all did not differ significantly.

In conclusion, the findings of this study prove that cloned caprine blastocyst could be produced via SCNT in Malaysia in which the caprine oocytes IVD competency post-SCNT was not compromised regardless of its resources from LOPU or abattoir and both intraSCNT and interSCNT approach possess more or less the same efficacy in producing cloned caprine blastocyst. With routine production of cloned caprine blastocyst via interSCNT using bovine oocytes as recipient cytoplast, it is hoped that in the near future, uterine embryo transfer could be integrated into caprine management practices for profitable and sustainable caprinecommercialisation particularly in Malaysia.

The authors wish to thank all the ABEL members in University of Malaya for their help and support. Heartiest thanks and gratitude is also attributed to Assoc. Professor Dr. RangsunParnpai (Suranaree University of Technology, Thailand) and Assist. Prof. DrMukesh K. Gupta (Konkuk University, South Korea) for their sharing of valuable knowledge. Appreciation is also addressed to the Shah Alam and Senawang Abattoir.

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EFFICACY OF CLONED CAPRINE EMBRYOS PRODUCTION USING INTRASPECIES- VERSUS INTERSPECIES SCNT APPROACH

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The success of somatic cell nuclear transfer (SCNT) technique to produce cloned animals was reported since 19th century. However, the success rate of SCNT applied on caprine is still low compared to other domestic animals like the bovine and ovine. Therefore there are still many areas of research that can be carried out to increase the success rate of caprine SCNT. At present, there are two possible approaches that can be applied to produce cloned embryos, intraspecies SCNT (intraSCNT) and interspecies SCNT (interSCNT). The application of interSCNT is known to play vital role in species preservation, livestock propagation and therapeutic cloning. Several studies have shown that ooplasm of bovine can support early development of embryos produced by nuclear transfer using somatic cell nuclei derived from different mammalian species such as sheep, pigs, rats (1), gaur (2), mouflon (3), panda (4), macaca (5), banteng (6) and monkey (7). Up to date, there is no report of cloned caprine embryo production via interSCNT approach. Therefore, this present study was conducted with the aim to produce cloned embryos in the caprine using intraSCNT versus interSCNT technique. As a control to this experiment, bovine intraSCNT was conducted. The source of bovine and caprine oocytes was obtained from the abattoir-derived ovaries and also via laparoscopy ovum pick-up (LOPU) technique on superovulated does. The collected oocytes were subsequently cultured in *in vitro* maturation medium for 18- 22 hours. The matured oocytes were then subjected to enucleation process. The bovine and caprine enucleated oocytes were then injected with either a male or female ear fibroblast cell from caprine or bovine. The couplets were electrofused and were cultured *in vitro* for the preimplantation development.

Both caprine intraSCNT and interSCNT approaches in this experiment enabled the production of cloned caprine embryos with the cleavage rate of 48.9% and 51.3% respectively. As for the bovine intraSCNT, the cleavage rate is significantly higher, 77.7% compared to the caprine intra- and interSCNT. At present, the cleaved cloned-caprine embryos using these two approaches managed to develop up to morula stage. In fact, researchers conducted cloning in caprine prefer to perform embryo transfer on early cell stages of *in vitro* derived caprine embryos as the *in vitro* developmental rate towards blastocyst often reduced drastically (8-9). Therefore, to evaluate the ability of the cloned-caprine embryos to develop beyond morula stage, perhaps embryo transfer at early cell stage might be a wise choice, while further modification on the activation and culture system of the cloned embryos will be carried out. In the nutshell, we have successfully cloned caprine embryos and both intraSCNT and interSCNT approach posses more or less the same efficacy.

The authors wish to thank all the ABEL members in University of Malaya and ESRC members in Suranaree University of Technology, Thailand for their help and support. Appreciation is also attributed to the Shah Alam and Senawang Abattoir. P.J.

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Appendix 3.2.3: **Kwong, P.J**., K. Sirattana, R. Parnpai, W.E. Wan Khadijah and R.B. Abdullah. 2009. *In vitro* production of cloned Gaur (*Bos gaurus*) blastocyst as an approach to conservation and domestication purposes. Proceedings of the 30th Annual Conference of Malaysian Society of Animal Production (MSAP), June 2-5, Hyatt Regency Hotel, Kota Kinabalu, Malaysia. pp. 43- 44 (Abstract).

Proc. Annu. MSAP Conf. 2 - 5 June 2009 Kota Kinabalu.

IN VITRO PRODUCTION OF CLONED GAUR (*Bos gaurus*) BLASTOCYST AS AN APPROACH TO CONSERVATION AND DOMESTICATION PURPOSES

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As a move to conserve gaur (*Bos gaurus*) from extinction and possible domestication for meat production, the application of assisted reproduction technology (ART) especially via reproductive cloning is gaining more attention by various laboratories worldwide. To date there are initial reports on the phenomenal research adapting interspecies somatic cell nuclear transfer (iSCNT) technique in rescuing highly endangered species like gaur (1), mouflon (2), panda (3), macaca (4) and banteng (5). The scarce oocytes resources of the endangered species had rendered the possibility of adapting autologous somatic cell nuclear transfer technique in propagating these endangered mammals. Therefore, iSCNT technique is ultimately the preferable technique used to cloned endangered species and currently, bovine oocytes are used extensively as recipient cytoplasm in this technique as Dominko *et al.* (6) had proven that the cytoplasm can support the development of introduced somatic cells of different donor cell species.

This study was conducted with the following objectives: to produce cloned gaur blastocyst using iSCNT technique, to evaluate the feasibility of producing cloned embryos using iSCNT approach and to observe the difference between the developmental rate of male and female cloned embryos. A total of 7 cloning replicates were carried out using bovine oocytes as recipient cytoplasts and both male and female gaur ear fibroblast cells as donor karyoplasts. The bovine oocytes were collected from abattoir derived ovaries and subsequently cultured *in vitro* for 22 hours. The matured oocyte was then subjected to enucleation process. The bovine enucleated oocytes were then injected with either a male or female gaur ear fibroblast cell. The couplets were electrofused and were cultured *in vitro* for the preimplantation development. The *in vitro* developments of the reconstructed embryos were consecutively observed and recorded at days-2, -4 and -8 post-activation.

A total of 191 *in vitro* matured oocytes at metaphase II were obtained. A reasonably high fusion percentage of enucleated recipient–karyoplast donor couplets was achieved (60.16 ± 4.08%). As for the percentage of cleaved reconstructed embryos, a total of 71.73 ± 3.91% of 2-cell embryos were obtained. Nevertheless, the percentage of blastocyst and hatched blastocyst obtained were only 19.75 ± 6.16% and 15.49 ± 5.66% respectively. Even though the percentages of both blastocyst and hatched blastocyst obtained in this study seem to be low, however, it is slightly higher as compared to the percentage reported by Lanza *et al.* (1). The study on the effect of donor cell gender showed that there were no significant differences in the percentage of *in vitro* development of male and female cloned embryos at all the stages of development. In

conclusions, the present study demonstrated that it is feasible to produce cloned gaur blastocyst via interspecies approach for both male and female donor karyoplasts

The authors wish to thank all the ESRC members in Sunranaree University of Technology, especially Miss Wanwisa Phewsoi and Mr. Sumeth Imsoonthornruksa for their help and guidance. The authors would like to express their gratitude as well to all the ABEL members in University of Malaya for their support. P.J. Kwong's research visit to ESRC was funded by PPP Research Fund (PS138/2008A) University of Malaya.

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Appendix 3.3: Proceeding (Poster Presentation)

Appendix 3.3.1: Abdullah R.B., **P.J. Kwong**, H.Y. Nam, W.E. Wan Khadijah and T. Kamarul. 2012. *In vitro* development of caprine embryos cloned with adult bone marrow mesenchymal stem cells. Proceeding of 9th Asian Reproductive Biotechnology Society (ARBS). October 23-27, Manila, Philippines. pp. 89 (Abstract)

P38

IN VITRO DEVELOPMENT OF CAPRINE EMBRYOS CLONED WITH ADULT BONE MARROW MESENCHYMAL STEM CELLS

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This study was undertaken to evaluate the possibility of using mesenchymal stem cells (MSCs) as donor karyoplasts to produce cloned goat embryos. At present, there is no report of using MSCs as donor karyoplasts for the production of cloned goat embryos. However, there were few reports of using bone-marrow MSCs as donor nuclei to produce cloned pig embryos, and their findings indicated that pig oocytes were capable to reprogramme MSCs. In this study, the caprine bone marrow aspirate was drawn from the iliac crest of the Boer goats.The mononuclear cells were then isolated by the Ficoll-Paque gradient density method. The resulted cell pellets were cultured in growth medium (low-glucose DMEM supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% Glutamax-1) (Invitrogen-Gibco, USA) in 75-cm2 tissue culture flasks. Fresh growth medium was replaced every 3 days until the cultures became 75% confluent. The cells were serially passaged, and expanded until passage-2 to be used as donor karyoplasts. Caprine oocytes were collected from abattoir and subjected to in vitro maturation (IVM) for 22-24 hours. The matured caprine oocytes were reconstructed with caprine MSCs and were cultured in vitro for 7 days in a modified KSOMaa culture medium system and the IVD rate of the embryos was recorded. The cleavage rate of reconstructed caprine oocytes obtained was 87.0±3.2%. These cloned caprine embryos managed to develop up to blastocyst hatched blastocyst stages with the rates of 19.67±3.4 and 16.54±2.98, respectively. In conclusion, cloned goat embryos using MSCs as donor karyoplasts could be produced in vitro.

IN VITRO DEVELOPMENT OF CAPRINE EMBRYOS CLONED WITH ADULT BONE MARROW MESENCHYMAL STEM CELLS

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1.0 INTRODUCTION

In Malaysia, caprine plays an important role as a provider of meat and milk sources as well as an animal model for biomedical research due to its less taboo connotations compared with bovine and porcine. Various ARTs were integrated into the conventional goat breeding programme to increase goat production. However, nuclear transfer (NT) is foreseen to facilitate the breeding programme efficiently by the propagation of elit traits in a short duration.

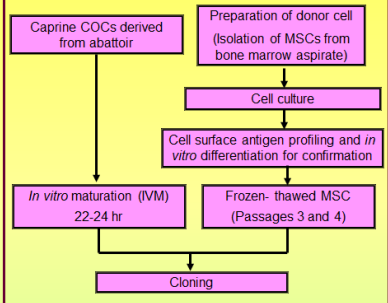
Various factors affecting NT efficiency were identified, including technical process, cell cycle stage, age of recipient oocyte and type of donor cell. The current available goat NT research mainly used either fœtal fibroblast cell or adult somatic cells as donor karyoplast (Baguisi *et al.*, 1999; Keefer *et al.*, 2002; Chen *et al.*, 2007). It has been suggested that in porcine and murine NT research, the genome of undifferentiated cells or partially differentiated multipotent progenitor cells can be easily reprogrammed by the recipient oocytes (Rideout *et al.*, 2000; Faast *et al.*, 2006; Zhu *et al.*, 2004) compared with other adult somatic cells. Thus in this study, the attempt of using undifferentiated cells (mesenchymal stem cell from bone marrow) as donor karyoplast was conducted with the aim to improve the cloned goat embryo production.

2.0 OBJECTIVE

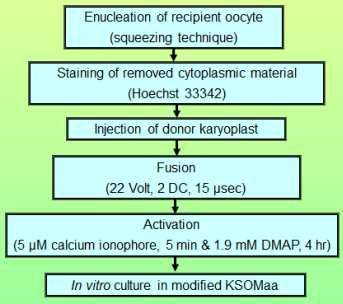
- To evaluate the efficacy of mesenchymal stem cell as donor karyoplast to produce cloned goat embryos

3.0 MATERIALS AND METHODS

3.2 Flow of experiment



3.3 Cloning method



3.4 Isolation and Characterisation of Caprine Mesenchymal Stem Cell

Figure 1: Caprine bone marrow aspirate were drawn from the iliac crest.

Figure 2: Mononuclear cells were isolated by the Ficoll-Paque gradient density method.

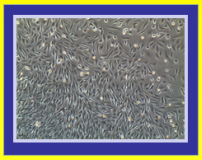


Figure 3:Primary culture of fibroblastic caprine mesenchymal stem cells (Magnification:4x).

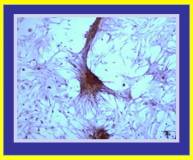


Figure 4: Mineralizing areas of cultures was shown after alizarin red staining (Magnification:10x).

3.5 Cloning procedure

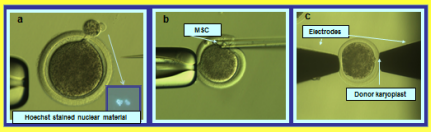


Figure 5: Cloning method- (a) Enucleation, (b) Donor cell injection, (c) Electrofusion.

4.0 RESULTS

Table 1: Percentage (mean ± SEM) of maturation, enucleation, and fusion rate for the production of caprine reconstructed oocytes using MSCs

	Percentage of		
	Maturation rate	Successful enucleation rate	Fusion rate
Manipulation efficiency	78.7 ± 0.9 (108/137)	86.67.1 ± 1.9 (94/108)	87.67 ± 4.4 (84/94)

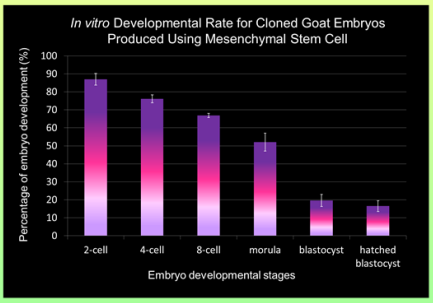


Figure 6: *In vitro* developmental rate of cloned goat embryos using mesenchymal stem cell as donor karyoplast.

In Vitro Development of Cloned Goat Embryos

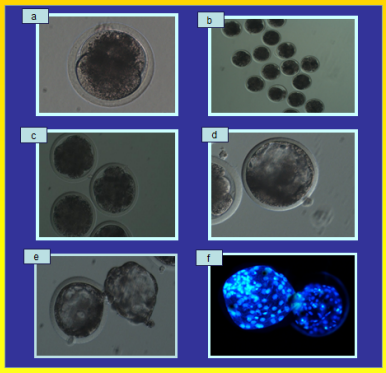


Figure 7: Cloned goat embryos - (a) 2-cell, (b) 4-cell, (c) 8-cell and Morula, (d) blastocyst, (e) hatched blastocyst, (f) hatched blastocyst stained with Hoechst 33342.

5.0 DISCUSSION

The efficiency of goat MSC to fuse with enucleated goat oocyte was comparable with the efficiency using skin fibroblast cell (83.7%) in our previous study (Kwong *et al.*, 2012) under the same fusion condition and parameters.

The cleavage rate of the MSC-derived cloned caprine embryos was comparable with the rate of MSC-derived cloned bovine and porcine embryos (Kato *et al.*, 2004; Lee *et al.*, 2010). However, the blastocyst and hatched blastocyst rates obtained in this study (19.7% and 16.5%) were relatively low compared to the study conducted by Kato *et al.* (2004) on bovine (39%) and Lee *et al.* (2010) on porcine (47.7%). In contrast, the blastocyst rate of MSC-derived cloned caprine embryos was found to be similar with cloned caprine embryos produced using foetal and skin fibroblast cell (Tang *et al.*, 2011;Kwong *et al.*, 2012) with the rate of (19% - 20.7%). This indicated that the *in vitro* development competency of late stage embryos for caprine species is still lacking compared to bovine and porcine, regardless of the donor cell type used in the caprine NT study.

The ability of the reconstructed caprine oocyte using MSC as donor karyoplast to develop into cloned embryos indicated that the undifferentiated MSC could be reprogrammed to re-activate the expression of early embryonic genes. In conjunction to this, the resulted blastocyst formation indeed can be used in future as a source of embryonic stem cell for regenerative medicine purposes and to produce live offspring for farming purposes.

6.0 CONCLUSION

The present study demonstrates that bone marrow mesenchymal stem cells can be isolated and identified from adult caprine and that adult mesenchymal stem cells have developmental totipotency after nuclear transfer.

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8.0 ACKNOWLEDGEMENTS

The authors wish to thank the ABEL and TEG members and staff of Institute Biological Sciences Mini Farm (Livestock), who helped in this project. This project was financially supported by HIR-MOHE project UM.C/HIR/MOHE/MED/04 Research Grant (PS367 / 2009A), University of Malaya.

Appendix 3.3.2: **Kwong, P.J**., H.H. Soh, W.E Wan Khadijah and R.B. Abdullah. 2012. Effect of donor cell types on *in vitro* developmental potential of caprine interspecies somatic cell nuclear transfer embryos. Proceeding of the 11th International Conference on Goats (IGA). September 24-27, Gran Canaria, Spain. pp. 402 (Abstract)

Reproduction, Artificial insemination

R-35

Effect of donor cell types on the in vitro developmental potential of caprine interspecies somatic cell nuclear transfer embryos

Kwong, P.J., H.H. Soh, W.E. Wan Khadijah, R.B. Abdullah

Animal Biotechnology-Embryo Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

This study aim to evaluate the effect of donor cell types namely, fetal fibroblast (FF) and ear fibroblast (EF) cell on the in vitro developmental (IVD) potential of cloned caprine embryos generated through caprine-bovine interspecies somatic cell nuclear transfer (iSCNT) approach. Bovine oocytes were collected from abattoir and subjected to in vitro maturation (IVM) for 22 - 24 hours. The matured bovine oocytes were allocated into 3 groups in which 2 groups will be reconstructed (cloned) with caprine FF and EF cells, respectively while the remaining group was parthenogenetically activated (PA) as a control group for this study. The cloned caprine embryos and PA embryos were cultured in vitro for 7 days in a modified KSOMaa culture medium system and the IVD rate of the embryos was recorded. The fusion rate of caprine FF cell with enucleated bovine oocyte (82.2%) was significantly ($P < 0.05$) higher compared to caprine EF cell with the bovine enucleated oocyte (74.6%). The rates of cleavage and development to the 4-cell and 8-cell stages for caprine iSCNT embryos derived from EF cell was significantly ($P < 0.05$) higher compared to caprine iSCNT embryos derived from FF cell. However, when approaching morula and blastocyst stage, the IVD competency of both EF- and FF-derived caprine iSCNT embryos did not differ significantly ($P > 0.05$). The blastocyst rates between PA embryos (12.7%) and EF- (9.0%) as well as FF- (13.5%) derived cloned embryos did not differ significantly ($P > 0.05$). In conclusion, both the caprine FF and EF cell had similar potential to support the development of caprine iSCNT embryos to the blastocyst stage.

EFFECT OF DONOR CELL TYPES ON *IN VITRO* DEVELOPMENTAL POTENTIAL OF CAPRINE INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

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1.0 INTRODUCTION

The emergence of various assisted reproduction technologies (ARTs) could offer promising opportunities in leveraging the platform to develop a sustainable goat farming industry to meet the yearly increasing demand of goat meat and dairy products besides sustaining the conventional breeding programme. Among the ARTs that are applied in goat farming industry, reproductive cloning technology via SCNT in production of cloned goat embryos is foreseen to facilitate the effort of mass goat production in just a short time frame. Furthermore, through the application of SCNT merged with molecular tools, it may bring the goat farming industry to a higher level technologies specifically the production of transgenic livestock with superior economic traits such as milk with medicinal property (Baldassarre and Karatzas, 2004).

However, in Malaysia, to achieve this goal of producing cloned-goat embryos using the intraSCNT is unattainable due to low source of goat oocytes as the recipient cytoplasts. Therefore, by using the cattle oocytes as recipient cytoplast in interSCNT (iSCNT) is the potential alternative approach to produce large number of cloned-goat embryos and subsequently offspring at a rapid rate. Thus this study was carried out to produce caprine-bovine iSCNT embryos and to evaluate which fibroblast cell types for efficient reprogramming by the bovine cytoplast.

2.0 OBJECTIVE

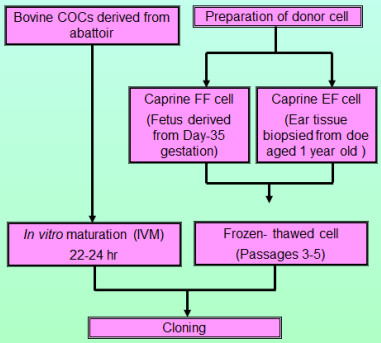
To evaluate the effect of donor cell types namely, fetal fibroblast (FF) and ear fibroblast (EF) cells on the *in vitro* developmental potential of cloned caprine embryos generated through caprine-bovine iSCNT approach.

3.0 MATERIALS AND METHODS

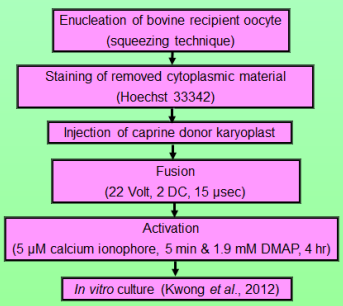
3.1 Experimental samples

a) Recipient cytoplast: bovine oocytes (Figure 1)
b) Donor karyoplast: caprine fetal fibroblast (FF) and ear fibroblast (EF) cells (Figure 2)

3.2 Flow of experiment



3.3 Cloning method



Experimental Animals and Samples Collection

Figure 1: Bovine cumulus oocyte complex.

Figure 2: Donor karyoplast: caprine fetal fibroblast and ear fibroblast cell.

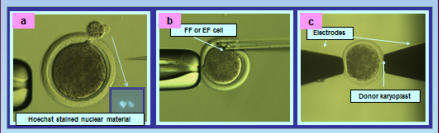
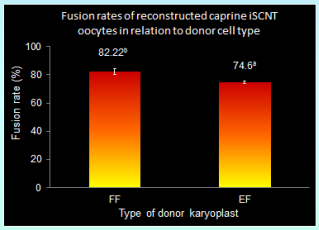


Figure 3: Cloning method- (a) Enucleation, (b) Donor cell injection, (c) Electrofusion.

4.0 RESULTS



ᵃ,ᵇ means with different superscripts were significantly different (P≤0.05).

Figure 4: Fusion rate of reconstructed caprine iSCNT oocyte in relation to donor cell type.

Table 1: Percentage (mean ± SEM) of *in vitro* developmental rate for caprine iSCNT embryos using two different donor cell types and bovine parthenogenesis embryos

Type of Embryo	Percentage				
	2-cell	4-cell	8-cell	Morula	Blastocyst
iSCNT-FF	61.7 ± 2.3ᵃ (88/146)	55.9 ± 2.2ᵃ (79/146)	39.5 ± 3.4ᵃ (56/146)	25.5 ± 3.0ᵃ (36/146)	13.5 ± 3.1ᵃ (18/146)
iSCNT-EF	77.9 ± 1.2ᵇ (129/165)	74.6 ± 2.0ᵇ (122/165)	63.7 ± 1.5ᶜ (104/165)	32.0 ± 2.0ᵃᵇ (54/165)	9.0 ± 0.4ᵃ (14/165)
PA	72.1 ± 2.5ᵇ (68/95)	62.3 ± 3.0ᵃ (59/95)	56.1 ± 1.7ᵇ (53/95)	35.3 ± 1.6ᵇ (33/95)	12.7 ± 2.0ᵃ (11/95)

ᵃ,ᵇ,ᶜ means with different superscripts in a column were significantly different (P≤0.05).
( ) = number of oocyte.

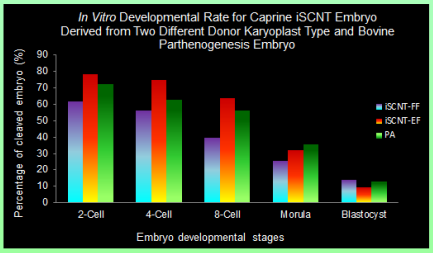


Figure 5: *In vitro* developmental rate of caprine iSCNT embryo derived from two different donor karyoplast type and bovine parthenogenesis embryo.

In Vitro Development of Caprine iSCNT Embryos

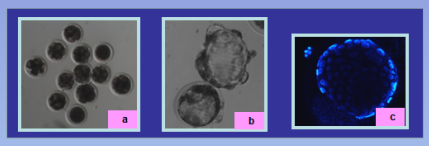


Figure 6: Caprine iSCNT embryos derived from fetal fibroblast cell- (a) 2 to 8-cell, (d) hatched blastocyst, (c) hatched blastocyst stained with Hoechst 33342.

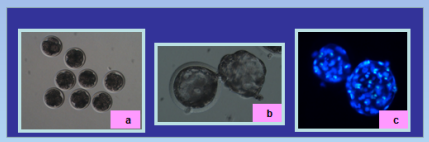


Figure 7: Caprine iSCNT embryos derived from ear fibroblast cell- (a) 2 to 8-cell, (d) hatched blastocyst, (c) hatched blastocyst stained with Hoechst 33342.

5.0 DISCUSSION

In this present study, the fusion rate of caprine reconstructed iSCNT embryos using FF cell was significantly higher (P≤0.05) compared to using EF cell. This was in contrast to the result obtained in cattle SCNT study conducted by Srirattana *et al.* (2010) in which, they demonstrated that the fusion rate of EF cell was significantly higher (P≤0.05) compared to FF cell under the same fusion parameter and medium in their study. On the other hand, Kato *et al.* (2000) demonstrated that in bovine SCNT study, fusion rate of FF cell (72-89%) was slightly higher compared to adult fibroblast (EF) cell (67-70%). Due to the variation in the fusion rate obtained in different studies, it is uncertain that the fusion rate in SCNT was affected by the type of fibroblast cell used as donor karyoplast. The overall fusion rate obtained in this caprine-bovine iSCNT study (74-82%) regardless of fibroblast cell type used as donor karyoplast was comparable to other iSCNT studies using bovine as recipient oocyte such as banteng (68-77%) by Sansinena *et al.* (2005) and buffalo (79-90%) by Srirattana *et al.* (2011).

Even though the fusion rate of reconstructed couplet using EF cell was lower, the cleavage rate and *in vitro* development (IVD) rate up to 8-cell stage for EF-derived iSCNT embryos was significantly higher (P≤0.05) compared to caprine iSCNT embryos reconstructed using FF cell. This trend of IVD was similar to bovine SCNT conducted by Kato *et al.* (2000) in which the cleavage rate of EF-derived SCNT embryos were relatively higher compared to FF-derived SCNT embryos.

However, at morula to blastocyst stages, the IVD competency of FF-derived iSCNT embryos seemed to be comparable to the EF-derived iSCNT embryos in this study. This results were similar to other cattle SCNT reports in which cloned cattle embryos derived from either FF or EF cells showed no difference in blastocyst formation rate (Hill *et al.*, 2000; Srirattana *et al.*, 2010). As reviewed by Loi *et al.* (2011), the blastocyst rate of caprine iSCNT embryos in this study was comparable to the results obtained in other caprine iSCNT studies (1.7- 7.4%) regardless of using FF or EF cell as donor karyoplast. This indicated that both fetal and adult fibroblast (EF) cell can be reprogrammed not only in an intraspecies cytoplast but also in an interspecies cytoplast.

6.0 CONCLUSION

Both the caprine FF and EF cells had similar potential to support the development of caprine iSCNT embryos to the blastocyst stage.

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8.0 ACKNOWLEDGEMENTS

The authors wish to thank the ABEL members and staff of Institute Biological Sciences Mini Farm (Livestock), who helped in this project. This project was financially supported by PPP Research Grant (PS367 / 2009A), University of Malaya.

Appendix 3.3.3: Abdullah, R.B., W.E Wan Khadijah, **P.J. Kwong** and H.H. Soh. 2011. Production of cloned caprine embryos through cumulus cell-whole cell intracytoplasmic injection and ear fibroblast cell-fusion approaches. Proceeding of the 15th Annual Conference of the European Society for Domestic Animal Reproduction. 2011. Turkey. Reproduction in Domestic Animals. Vol 46, Supplement 3. pp. 78 (Abstract)

Reprod Dom Anim **46** (Suppl. 3), 78–161 (2011); doi: 10.1111/j.1439-0531.2011.01839.x

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Poster Abstracts

P1

Effects of L-NAME (a nitricoxide synthase inhibitor) on *in vitro* maturation of sheep oocytes

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Nitricoxide (NO) is a biological signaling molecule that is generated by NO synthase (NOS) from L-arginine. It has been demonstrated that NO has a crucial role in maturation of mammalian oocytes. In this study, the importance of NO/NOS system in in-vitro maturation of ovine oocytes was investigated. Different concentrations (0.1, 1, 10 mM) of L-NAME, a NOS inhibitor, were used to evaluate the effect of the inhibition of NOS on cumulus expansion and meiotic resumption of sheep oocytes. The results were evaluated by chi-square test and $p < 0.05$ was considered significant. L-NAME in the highest concentration (10 mM) inhibited total cumulus expansion as compared to control ($p < 0.05$). It also suppressed the meiotic maturation and the extrusion of first polar body in a dose-dependent manner. The percentage of oocytes at MII stage was 26.47%, 40.72%, 63.78% and 75.16% for 10, 1, 0.1 mM and control group, respectively. To evaluate if the effect is reversible, 0.1 mM sodiumnitroprusside (SNP, a NO donor) was added in the treatment containing 10 mM L-NAME. The concomitant addition of L-NAME with SNP reversed the inhibitory effect of L-NMAE on cumulus expansion and meiotic maturation. These results indicate that NO/NOS system is involved in maturation of sheep oocytes.

P2

Total lymphocyte counts are affected by *Neospora canium* during the peripartum period in dairy cows

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Neosporosis is a protozoan-parasitic disease that affects most warm-blooded animals. Bovine neosporosis is characterised by high rates of mid-gestational abortion. Aiming to study the effect of neosporosis on the peripartum immunity, 612 blood samples of 85 Neospora-seronegative and 17 Neospora-seropositive high-producing dairy cows were collected every 2 weeks during the last 2 months of gestation and the 1st week postpartum (six samples/animal). Total and differential leukocyte counts were automatically analysed using HEMA-VET®. Blood counts were analysed by repeated measures GLM ANOVA in regard to Neospora-seropositivity. Lymphocytes in Neospora-seropositive animals were significantly lower than in Neospora-seronegative animals on the first sample at 8th pre-partum week but increased and showed a peak (reaching seronegative animal's level) on the 4th pre-partum week ($p = 0.049$; within-subject effect). Meanwhile, Lymphocytes in Neospora-seronegative cows showed a slight decline during the pre-partum period. No postpartum differences were found among the two groups. During late gestation, the maternal immune system is recovering from the immune-depression of the second gestation-term. Lymphocytes peak observed at the 4th pre-partum week in Neospora seropositive cows would suggest that the immune response was highly activated during a punctual period of time during the third term of gestation compared to seronegative cows.

P3

Production of cloned caprine embryos through cumuls cell-whole cell Intracytoplasmic injection and ear fibroblast cell-fusion approaches

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At present, research on the production of cloned caprine embryos and offspring at global level is still not as advanced compared to other domestic animals such as bovine and ovine. Thus there are many factors that can be refined to improve the success rate of cloned caprine embryo production. The objective of the study was to evaluate some of the factors affecting the production of cloned caprine embryos *in vitro*. The factors were combination of cloning technique, type of donor cell and as well as the *in vitro* culture (IVC) medium. Combinations investigated were caprine cumulus-cell-whole cell intracytoplasmic cell injection-mSOF medium (CC-WCICI-mSOF), caprine ear fibroblast cell-fusion-mSOF (EF-F-mSOF) and caprine ear fibroblast cell-fusion-KSOM (EF-F-KSOM). The *in vitro* developments of the reconstructed oocytes were evaluated from day 2 post-activation to day 9. The cleavage rate of reconstructed oocyte in experiment EF-F-KSOM and EF-F-mSOF were higher compared to CC-WCICI-mSOF (82.23%, 69.63% and 13.51% respectively). Of the reconstructed oocytes in experiment EF-F-KSOM 5.92% managed to develop up to blastocyst, while no blastocyst was obtained using EF-F-mSOF or CC-WCICI- mSOF. The combination of EF-F-KSOM factors enables the production of caprine blastocyst. In conclusion, it is possible to produce cloned caprine embryos using both approaches; however in this experiment, KSOM medium is favourable to produce caprine cloned blastocyst.

PRODUCTION OF CLONED CAPRINE EMBRYOS THROUGH CUMULUS CELL-WHOLE CELL INTRACYTOPLASMIC INJECTION AND EAR FIBROBLAST CELL-FUSION APPROACHES

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1.0 INTRODUCTION

Production of viable cloned animals such as bovine (Cibelli et al., 1998), murine (Wakayama et al., 1998), caprine (Baguisi et al., 1999) and porcine (Polejaeva et al., 2000) has been accomplished using somatic cell nuclear transfer (SCNT) approach ever since the birth of first cloned sheep, 'Dolly'. However, the overall success rate of SCNT-derived blastocysts is still low at present. In addition, the percentage of success rate reported by different laboratories often varies in each species.

The success rate of SCNT at the level of embryo production is known to be affected by various factors such as (i) manipulation technique [fusion vs. intracytoplasmic injection (ICI)], (ii) type of donor karyoplast, (iii) type of recipient ooplasm, (iv) type of activation treatment and (v) *in vitro* culture (IVC) system. There were no laboratories that can proclaim that which combination of manipulation techniques, donor karyoplast, activation treatment and IVC medium is the best as each laboratories under their settings. Therefore in our laboratory, there were three combinations of approach utilised in producing cloned caprine embryos, namely (i) ear fibroblast cell- fusion-potassium simplex optimized medium (EFC-Fusion-KSOM), (ii) EFC-Fusion-modified synthetic oviductal fluid (EFC-Fusion-mSOF) and cumulus cell-whole cell intracytoplasmic injection - mSOF(CC-WCICI-mSOF).

2.0 OBJECTIVES

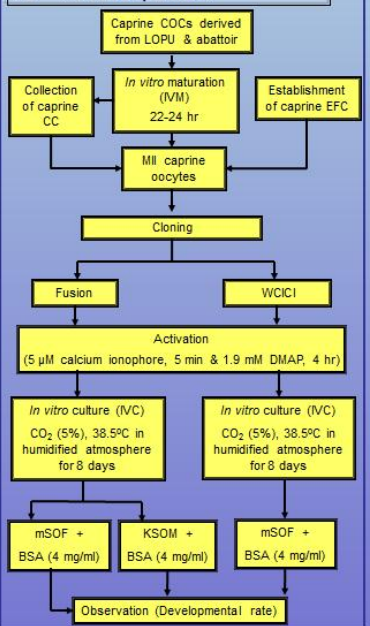
a) To produce cloned caprine embryos via SCNT.
b) To evaluate the efficacy of three different SCNT approaches (i) EFC-Fusion-KSOM, (ii) EFC-Fusion-mSOF, (iii) CC-WCICI-mSOF.

3.0 MATERIALS & METHODS

3.1 Experimental samples

a) Recipient cytoplast: caprine oocytes.
b) Donor karyoplast: caprine cumulus cells (CC) and ear fibroblast cells (EFC)

3.2 Flow of experiment



3.3 Cloning method

1 Enucleation

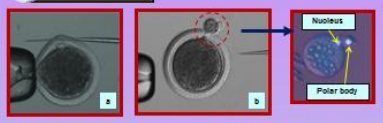


Figure 1 (a-b): Enucleation via squeezing technique.

Figure 2: Stained nuclear material with Hoechst 33342.

2a Fusion

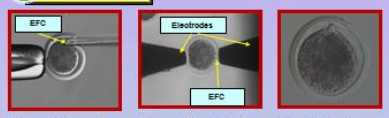


Figure 3: Subzonal injection of caprine EFC.

Figure 4: Electrofusion (20-21 Volt, 2 DC, 15 µsec).

Figure 5: Fused couplet.

2b WCICI

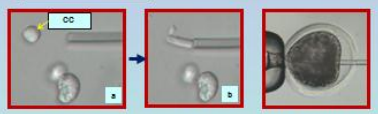


Figure 6 (a-b): Removal of plasma membrane of caprine cumulus cell.

Figure 7: Injection of CC into enucleated caprine ooplasm.

4.0 RESULTS

Table 1: Percentage (mean ± SEM) of *in vitro* developmental rate for cloned caprine embryos manipulated using 3 different approaches

Treatment	*In vitro* developmental rate (IVD)				
	2-cell	4-cell	8-cell	Morula	Blastocyst
EFC-Fusion-KSOM	79.0±3.2^{a,y} (183/233)	71.4±3.8^{a,x} (164/233)	62.9±3.5^{a,x} (141/233)	40.7±2.8^{a,x} (93/233)	15.9±3.8^{a,y} (29/233)
EFC-Fusion-mSOF	70.2±4.8^{a,y} (133/191)	53.9±3.1^{b,y} (102/191)	41.7±4.0^{b,y} (78/191)	21.6±6.7^{b,y} (40/191)	0.0±0.0^{a,x} (0/191)
CC-WCICI-mSOF	11.5±5.4^{b,x} (5/37)	1.4±1.4^{a,x} (1/37)	0.0±0.0^{a,x} (0/37)	0.0±0.0^{a,x} (0/37)	0.0±0.0^{a,x} (0/37)

^{a,b,c,d} means with different superscripts in a row were significantly different (P≤0.05).
^{x,y,z} means with different superscripts in a column were significantly different (P≤0.05).
( ) = number of embryos.

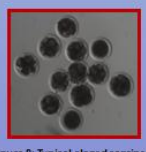


Figure 8: Typical cloned caprine 4 to 8-cell embryos.

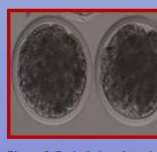


Figure 9: Typical cloned caprine morula.

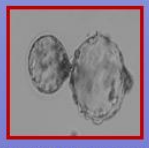


Figure 10: Typical cloned caprine hatched blastocyst.

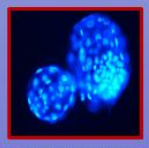


Figure 11: Fluorescent stained cloned caprine hatched blastocyst.

5.0 DISCUSSION

In this present study, all the 3 approaches of SCNT were successful in producing doned caprine embryos. However, the EFC-Fusion-KSOM gave better cleavage rate compared to EFC-Fusion-mSOF and CC-WCICI-mSOF, particularly from 4-cell stage onwards (P≤0.05). Furthermore, the ability of EFC-Fusion-KSOM approach in supporting the cloned caprine blastocyst production showed that, IVC medium did affect the efficacy of SCNT. However, the outcome of each IVC medium in supporting embryo development were known to vary among laboratories due to presence of heterogenous sub-factors such as nutrient supplementation, proteins and incubation environment. For instance, Tang et al. (2011) reported that fetal fibroblast derived cloned caprine embryos were able to develop to blastocyst stage in mSOFBSA medium which is in contrast to our result. However, supplementation of 10% FBS was done at 72 hr in their study and perhaps this might be the factor why in our study cloned caprine blastocyst could not develop in mSOF basal culture medium. On the other hand, cloned caprine embryos could develop to blastocyst in KSOM IVC medium as shown in our finding that could be due to the presence of EDTA as inhibitor of reactive oxygen species (Johnson et al., 1994) and addition of glucose which is needed by later stages of caprine embryos for the high metabolic requirement.

The CC-WCICI-mSOF approach was conducted as a preliminary study as reflected in low IVD rate at all cell stages of development. Therefore, comparison between the treatment approaches is relatively inconclusive. This is believed to be due to the small sample size and the learning curve stage for the establishment of WCICI approach. Nevertheless, the data presented here is impressive with development of embryos using cumulus cell as donor karyoplast and WCICI as the SCNT procedure as an alternative and comparative approach to produce cloned caprine embryos for future research. Previously, Chen et al. (2007) and Lee et al. (2003) reported that by using WCICI technique, cloned caprine and porcine blastocyst were able to be produced (8.8% and 48% respectively). Thus, we believe if further modification in the CC-WCICI-mSOF approach could be carried out in future, it might enable cloned caprine blastocyst to be produced using this approach.

6.0 CONCLUSION

Cloned caprine embryos can be produced using all 3 approaches (EFC-Fusion-KSOM, EFC-Fusion-mSOF and CC-WCICI-mSOF). However, the efficacy of producing cloned caprine blastocyst apparently better when reconstructed caprine embryos using EFC were cultured in KSOM based IVC medium in our laboratory. However more studies are needed for CC-WCICI-mSOF approach to corroborate the findings of this present research.

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8.0 ACKNOWLEDGEMENTS

The authors wish to thank the ABEL members and staff of Institute of Biological Sciences Mini Farm (Livestock), who helped in this project. This project was financially supported by PPP Research Grant (PS425/2010A and PS288/2010A), University of Malaya.

15th Annual Conference of European Society for Domestic Animal Reproduction ESDAR (15-17th September 2011)

Appendix 3.3.4: **Kwong, P.J**., W.E. Wan Khadijah and R.B. Abdullah. 2010. Effect of 2 different IVM intervals on ovarian hyperstimulated goat oocyte developmental competency post-SCNT. Proceeding of the 7th Annual conference of the Asian Reproductive Biotechnology Society (ARBS). November 8-12, Kuala Lumpur, Malaysia. pp. 104 (Abstract)

P 71

EFFECT OF 2 DIFFERENT IVM INTERVALS ON OVARIAN HYPERSTIMULATED GOAT OOCYTE DEVELOPMENTAL COMPETENCY POST-SCNT

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Generally, the optimal *in vitro* maturation (IVM) duration for goat oocytes was reported to be between the range of 24-27 hr in any research pertaining to the *in vitro* production of goats embryos up to date. However the source of goat oocytes that were being used in these reports generally from abattoir derived ovaries where does were non-synchronized and non-superovulated. Therefore, the aim of this study was to determine the effect of two different IVM intervals (23-27 hr and 18-22 hr) on the ovarian hyperstimulated goat oocytes *in vitro* developmental competency after nuclear transfer. Goats were estrus synchronized with an intravaginal insert containing 0.3 g progesterone (CIDR) for 14 days followed by luteolytic treatment with the administration of 125 µg cloprostenol 36 hr prior CIDR removal. The does were subjected to ovarian hyperstimulation with 70 mg FSH and 500 IU LH given via intra-muscular (i.m.) injection 60-72 hr prior laparoscopic ovum pick-up (LOPU). A total of 18 does with the yield of 101 and 83 cumulus oocytes complexes (COCs) were being subjected to IVM duration of 23-27 hr and 18-22 hr, respectively. The matured oocytes derived from these 2 IVM duration treatments were being used as the recipient cytoplast for the somatic cell nuclear transfer experiment. The *in vitro* developmental rate of oocytes derived from the 2 IVM duration treatments were evaluated. The maturation rates of COCs derived from 18-22 hr IVM duration (75.21%) were significantly higher compared to the 23-27 hr IVM duration (61.38%). The same trend were observed in the cleavage rate as the reconstructed oocytes from 18-22 hr IVM duration (84.23%) were significantly higher compared to the 23-27 hr IVM duration (67.74%). The *in vitro* developmental rate of reconstructed oocyte to morula stage for 18-21 hr IVM duration group (46.58%) is significantly higher compared to the 23-27 hr IVM duration group (24.51%). Both reconstructed oocytes from 18-21 hrs and 23-27 hr IVM duration group portray a significant reduction in the developmental rate from 8-cell to morula. The *in vitro* developmental rate of reconstructed oocytes were compared up to morula stage as both group of oocytes failed to develop beyond morula stage. In conclusion, the results in our study shown that, the IVM duration of 18-22 hr gave a better oocytes developmental competency for oocytes derived from ovarian hyperstimulated does.

EFFECT OF TWO DIFFERENT IVM INTERVALS ON OVARIAN HYPERSTIMULATED GOAT OOCYTE DEVELOPMENTAL COMPETENCY POST-SCNT

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1.0 INTRODUCTION

At present, the optimal *in vitro* maturation (IVM) duration for goat oocytes was reported to be between the range of 24-27 hr in any research pertaining to the *in vitro* production of goats embryos. However the source of goat oocytes that were being used in these reports generally from abattoir derived ovaries where does were non-synchronized and non-superovulated. There might be a variation in the IVM duration for oocytes retrieved from oestrus synchronised and superovulated does as compared to the non-hormonal treated does. In this research, a standard hormonal regime adapted from Abdullah *et al.*, 2008 which emphasis on the prolonging of the interval from ovarian hyperstimulation to Laparoscopic Ovum Pick Up (LOPU) technique was applied to oestrus synchronise and superovulate. The oocytes retrieved were used to test on the effect of 2 different IVM intervals which are 18 -22 hour and 23 -27 hour on the maturation rate and developmental rate of the goat oocytes after somatic cell nuclear transfer.

IVM is indeed an important factor that will influence the subsequent in vitro development of oocytes to embryos. Therefore, an optimum IVM duration need to be determine.

2.0 OBJECTIVE

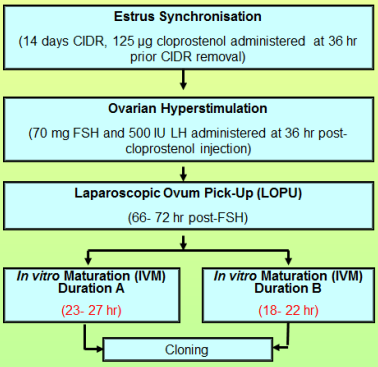
a) To determine the effect of two different IVM intervals (23- 27 hr and 18- 22 hr) on the ovarian hyperstimulated goat oocytes *in vitro* developmental competency after nuclear transfer.

3.0 MATERIALS AND METHODS

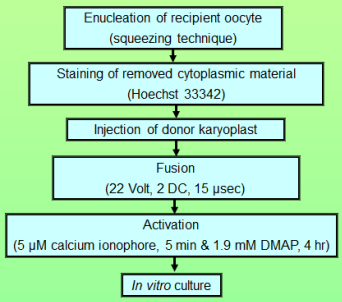
3.1 Experimental animals

a) Recipient cytoplast: Boer goat oocytes (Figure 1)
b) Donor karyoplast: Jermasia ear fibroblast cell (Figure 2)

3.2 Flow of experiment



3.3 Cloning method



Experimental Animals and Samples Collection

Figure 1: Boer goat oocyte.

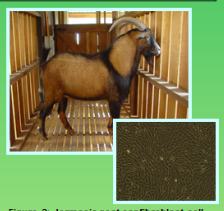


Figure 2: Jermasia goat ear fibroblast cell

Figure 3: Goat oocyte retrieval via LOPU.

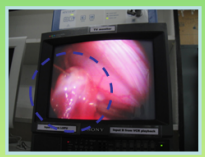


Figure 4: Closed up image of the ovary during LOPU.

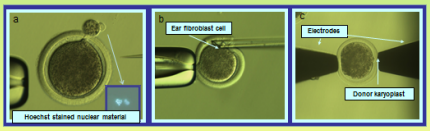


Figure 5: Cloning method- (a) Enucleation, (b) Donor cell injection, (c) Electrofusion.

4.0 RESULTS

Table 1: Percentage (mean ± SEM) of maturation, enucleation, fusion and cleavage rate of LOPU derived goat oocyte treated in 2 different IVM intervals

IVM Duration (Hour)	Percentage of			
	Maturation rate	Successful enucleation rate	Fusion rate	Cleavage rate
18 – 22	75.2 ± 3.3^{b} (63/83)	78.1 ± 4.6^{a} (50/63)	82.5 ± 3.1^{a} (41/50)	84.2 ± 5.5^{b} (34/41)
23 – 27	61.4 ± 1.5^{a} (62/101)	71.6 ± 3.5^{a} (44/62)	84.1 ± 4.5^{a} (37/44)	67.7 ± 1.7^{a} (25/37)

^{a,b} means with different superscripts in a column were significantly different (P≤0.05).
( ) = number of oocyte.

Table 2: Percentage (mean ± SEM) of *in vitro* developmental rate for cloned goat embryos manipulated using LOPU derived oocytes matured in 2 different IVM intervals

IVM Duration (Hour)	Percentage of			
	2-cell	4-cell	8-cell	Morula
18 – 22	$84.2 \pm 5.5^{z,b}$ (34/41)	$74.7 \pm 4.1^{xy,b}$ (30/41)	$65.9 \pm 6.7^{y,b}$ (26/41)	$46.6 \pm 4.4^{x,b}$ (19/41)
23 – 27	$67.7 \pm 1.7^{z,a}$ (25/37)	$60.4 \pm 4.6^{xy,a}$ (22/37)	$46.4 \pm 3.4^{y,a}$ (16/37)	$24.5 \pm 7.0^{x,a}$ (9/37)

^{a,b} means with different superscripts in a column were significantly different (P≤0.05).
^{x,y,z} means with different superscripts in a row were significantly different (P≤0.05).
( ) = number of oocyte.

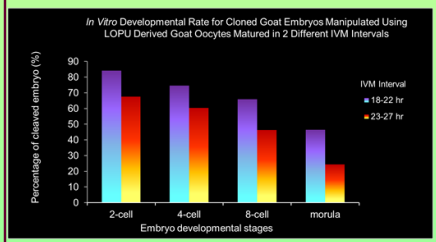


Figure 7: *In vitro* developmental rate of cloned goat embryos using oocytes matured in 2 IVM intervals.

Morphology of Goat Matured Oocyte

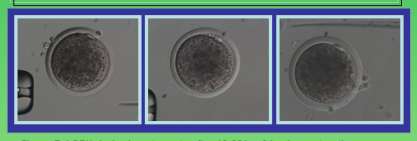


Figure 7: LOPU derived goat oocyte after 18-22 hr of *in vitro* maturation

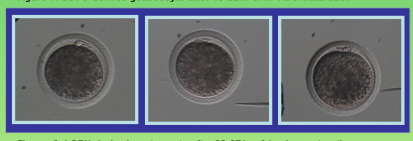


Figure 8: LOPU derived goat oocyte after 23-27 hr of *in vitro* maturation

In Vitro Development of Cloned Goat Embryos

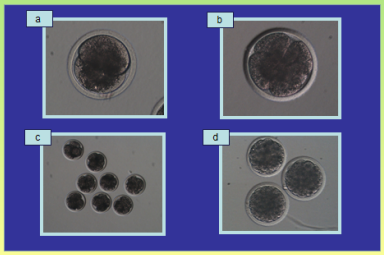


Figure 9: Cloned goat embryos - (a) 2-cell, (b) 4-cell, (c) 8-cell, (d) Morula

5.0 DISCUSSION

In this present study, LOPU derived oocytes matured in IVM duration of 18-22 hours seems to give a better maturation rate and cleavage rate compared to 23-27 hours (75.2% vs 61.4%) and (84.2% vs 67.7%) respectively. The IVM process of oocytes might have begin to occur in the follicles of the superovulated doe ovary due to the prolonging of ovarian hyperstimultion and LOPU interval regime (66-72 hours) that we applied in this research. Therefore the duration of IVM required for oocytes retrieved from the does using our hormonal regime is shorter. However, Baldassarre *et al.*, 2002 reported that IVM interval of 24-27 hours is optimum for LOPU derived oocytes in their experiment as they adapted a shorter ovarian hyperstimulation and LOPU interval regime (48 hours).

The developmental rate of cloned embryos derived from oocytes treated in IVM duration of 18-22 hours is better compared to the 23-27 hours. However, the *in vitro* development of the cloned embryos only manage to develop up to morula stage. There are reports of *in vitro* development of cloned goat embryos up to blastocyst stage by other researchers as generally the IVC system adapted in their research (Keefer *et al.*, 2002) using culture condition of 5% CO_2, 5% O_2,and 90% N_2 at 38.5°C. This might be the factor that contribute to the results obtained in this study as embryos fail to develop to blastocyst. Generally, caprine embryos are known to have a lower *in vitro* developmental potential towards the late perimplantation stage. Even the reports of success in producing cloned kids involved the transfer of embryos at early cloned embryos stages from 2- 8 cell stages. Therefore an optimum IVC system must be develop and improved in this study for cloned goat embryos (Baguisi *et al.*, 1999).

6.0 CONCLUSION

In conclusion, the results in our study shown that, the IVM duration of 18-22 hr gave a better developmental competency for oocytes derived from ovarian hyperstimulated does post-SCNT.

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8.0 ACKNOWLEDGEMENTS

The authors wish to thank the ABEL members and staff of Institute Biological Sciences Mini Farm (Livestock), who helped in this project. This project was financially supported by PPP Research Grant (PS367/ 2009A), University of Malaya.

Appendix 3.3.5: Abdullah, R.B., **P.J. Kwong** and W.E. Wan Khadijah. 2009. Production of cloned caprine embryos through interspecies somatic cell nuclear transfer approach. Proceeding of the 6th Annual conference of the Asian Reproductive Biotechnology Society (ARBS). November 16-20, Siem Reap, Cambodia. pp. 59 (Abstract)

P 10

PRODUCTION OF CLONED CAPRINE EMBRYOS THROUGH INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER APPROACH

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Since 1930s, remarkable discoveries were obtained in the field of animal reproductive cloning. A dramatic research transition field from nature's ancient unassisted cloning to the modern era attempt to clone mammals in the laboratory has generated the discoveries of the possibility in producing viable cloned offspring through somatic cell nuclear transfer technique (SCNT). At presents, both intraspecies (intraSCNT) and interspecies somatic cell nuclear transfer technique (interSCNT) approaches are being applied to produce various cloned animals like ovine, bovine, caprine, murine and porcine. There were several reports on the successful production of cloned caprine offspring via intraSCNT approach (Baguisi *et al.*, 1999; Keefer *et al.*, 2002 and Chen *et al.*, 2007). However, to date there are still no reports of successful cloned kids produced through interSCNT using bovine oocyte as recipient cytoplasm. Dominko *et al.* (1999) had proven that the bovine cytoplasm can support the development of introduced somatic cells derived from different donor cell species. This interSCNT approach is indeed useful to enable production of cloned embryos in circumstances where scarce oocyte resources of the domestic goats arise. Today, interSCNT using bovine oocyte as recipient cytoplasm is not being integrated solely to mass propagate superior animal for commercialisation purposes. Indeed its importance and potentials are foreseen to facilitate the conservation effort of endangered animals like gaur and banteng (Lanza *et al.*, 1999; Sansinena *et al.*, 2005). Therefore, in this present study, bovine oocyte is being integrated as the recipient cytoplasm to produce cloned caprine embryos with the futuristic view that this approach can be applied to save extinct caprine species like the serrow goat. This study was conducted with the aim to produce cloned caprine embryos using interSCNT technique. The bovine oocytes were collected from abattoir derived ovaries and subsequently cultured *in vitro* for 22 hours. The matured oocyte was then subjected to enucleation process. The enucleated bovine oocytes were then injected with either a male or female caprine ear fibroblast cell. The couplets were electrofused and were cultured *in vitro* for the preimplantation development. The *in vitro* developments of the reconstructed embryos were consecutively observed and recorded at days 2, -4 and -8 post-activation. In this study, approximately 60% of *in vitro* matured bovine oocytes at metaphase II were obtained. As for the *in vitro* development of the reconstructed embryos, embryonic developmental arrest at the 8-cell stage was observed. This similar outcome was reported by Sansinena (2004) and Tao *et al.* (2008) in the production of cloned caprine embryos through interSCNT. In conclusion, the present study demonstrates that it is possible to produce cloned caprine embryos through interSCNT. However, continuous study will be carried out to breakthrough the embryonic developmental arrest of the cloned embryos at 8-cell stage.

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PRODUCTION OF CLONED CAPRINE EMBRYOS THROUGH INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER APPROACH

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1.0 INTRODUCTION

Since 1930s, remarkable discoveries were obtained in the field of animal reproductive cloning. A dramatic research transition field from nature's ancient unassisted cloning to the modern era attempt to clone mammals in the laboratory has generated the discoveries of the possibility in producing viable cloned offspring through somatic cell nuclear transfer technique (SCNT). At presents, both intraspecies (intraSCNT) and interspecies somatic cell nuclear transfer technique (interSCNT) approaches are being applied to produce various cloned animals like ovine, bovine, caprine, murine and porcine. There were several reports on the successful production of cloned caprine offspring via intraSCNT approach (Baguisi *et al.*, 1999; Keefer *et al.*, 2002 and Chen *et al.*, 2007). However, to date there are still no reports of successful cloned kids produced through interSCNT using bovine oocyte as recipient cytoplasm. This interSCNT approach is indeed useful to enable production of cloned embryos in circumstances where scarce oocyte resources of the domestic goats arise.

Today, interSCNT using bovine oocyte as recipient cytoplasm is not being integrated solely to mass propagate superior animal for commercialisation purposes. Indeed its importance and potentials are foreseen to facilitate the conservation effort of endangered animals like gaur and banteng (Lanza *et al.*, 1999; Sansinena *et al.*, 2005). Therefore, in this present study, bovine oocyte is being integrated as the recipient cytoplasm to produce cloned caprine embryos with the futuristic view that this approach can be applied to save extinct caprine species like the serrow goat.

2.0 OBJECTIVE

a) To produce cloned caprine embryos via interspecies SCNT approach.

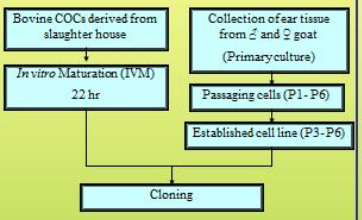
3.0 MATERIALS AND METHODS

3.1 Experimental animals

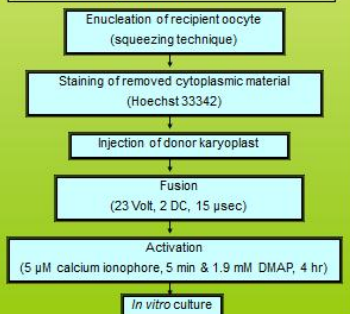
a) Recipient cytoplast: bovine oocytes (Figure 1)

b) Donor karyoplast: caprine ear fibroblast cell (Figure 2)

3.2 Flow of experiment



3.3 Cloning method



Experimental animals and samples

Figure 1: Bovine where source of oocytes were retrieved.

Figure 2: Caprine ear tissue explant was biopsied.

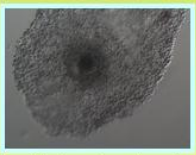


Figure 3: Bovine cumulus oocyte complexe.

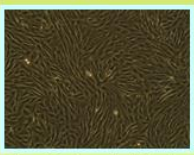


Figure 4: Caprine ear fibroblast cells.

Cloning technique

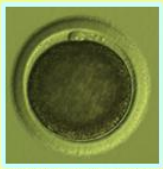


Figure 5: Metaphase II bovine oocyte.

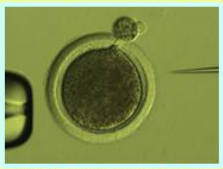


Figure 6: Enucleation via squeezing technique.

Figure 7: Injection of goat ear fibroblast cell into enucleated oocyte.

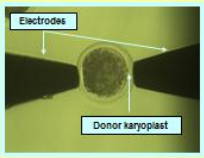


Figure 8: Fusion

4.0 RESULTS

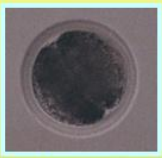


Figure 9: Cleaved reconstructed oocyte (2- cell)

Figure 10: Cleaved reconstructed oocyte (8- cell)

Figure 11: Cleaved reconstructed oocyte (8-cell)

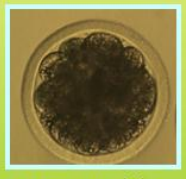


Figure 12: Cleaved reconstructed oocyte (morula)

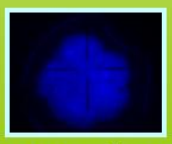


Figure 13: Fluorescent stained 8- cell

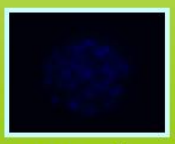


Figure 14: Fluorescent stained morula

5.0 DISCUSSION

The progress of this study is still at the preliminary stage as the overall percentage of cleaved reconstructed oocytes obtained in this study is still low approximately 2- 3%. Only one reconstructed embryos manage to develop to morula. Analysis of the data could not be carried out due to the insufficient number of replicate available.

There were a few factors influences the outcome of this study at present. The quality of bovine oocytes obtained from the abattoir is one of the factor. Poor quality bovine oocytes were obtained frequently and as a result, inconsistent maturation percentage was obtained. The maturation percentage obtained up to date ranged from 20% to 60%. A higher maturation percentage were obtained from cumulus oocyte complexes (COCs) with homogenous cytoplasmic distribution compared to COCs with heterogenous cytoplasmic distribution. According to Sirard *et al.* (2006), the quality of bovine oocytes do affect the ability of oocytes in the resumption of the meiosis process and developmental potential of embryos. Therefore the low maturation rate obtained might be pertaining to that reason.

Futhermore, at present, there are still many unanswered queries pertaining to the effect on mitochondrial incompatibilities or epigenetic factors that might affect the nuclear-ooplasmic events occuring at the time of genomic activation, especially on interSCNT derived cloned goat embryos. Researches on cloned goat via interSCNT were carried out by Sansinena (2004) and Tao *et al.* (2008) and no blastocyts were reported in their study. Therefore further study on the molecular level regarding the distance of caprine and bovine lineages could be carried out in future to see the possibility of incorporating bovine oocytes as recipient cytoplast and caprine as donor karyoplast in interSCNT.

6.0 CONCLUSIONS

Cleaved reconstructed oocytes were obtained in this preliminary stage study. However, the percentage is still low and further study on the molecular mechanism and also improvement in the activation treatment will be done to enhance the efficiency in producing cloned caprine embryos via InterSCNT approach.

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